MAJOR ARTICLE

Recent and Rapid Emergence of W-Beijing Strains of *Mycobacterium tuberculosis* in Cape Town, South Africa

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Background. There is increasing evidence of a strain-related variation in the virulence in *Mycobacterium tuberculosis* that may afford a selective advantage to certain strains. The W-Beijing strain family is globally distributed, highly virulent in animal models, associated with human immunodeficiency virus infection and drug resistance, and may be an emerging strain family. Our goal was to determine whether W-Beijing strains are expanding in a region of South Africa where rates of tuberculosis are among the highest in the world.

Methods. We used spoligotyping and single nucleotide polymorphism analysis to genotype all strains of tuberculosis from children presenting to the major pediatric referral hospital in Cape Town, South Africa over a period of 4 years and strains present in 352 archived histological samples from over a 76-year period.

Results. The proportion of W-Beijing strains from children increased from 13% to 33% from 2000 to 2003 ($P = .026$). With regard to the histological samples, W-Beijing strains were absent in the samples from the period 1930–1965 and rare in the samples from the period 1966–1995 (2.8% of samples), but they were increasingly common in samples from the period 1996–2005 (20% of samples; $P = .001$).

Conclusions. The rapid expansion of W-Beijing strains in a region with a very high background incidence of tuberculosis suggests that these strains have a significant selective advantage. The biological reasons for this observation remain unclear but warrant further study. The rapid spread of this virulent strain lineage is likely to present additional challenges for tuberculosis control.

The W-Beijing family of strains of *Mycobacterium tuberculosis* is globally distributed [1] and documented as a cause of outbreaks of infection that often involve multidrug-resistant organisms [2]. W-Beijing strains have been associated with extrathoracic disease [3] and HIV infection [4]. In experimental animal models, W-Beijing strains were highly virulent [5, 6], and animals were not protected by prior bacille Calmette-Guérin vaccination [7]. Evidence suggesting that these strains are emerging in several regions is, therefore, of concern [8]. However, such evidence is limited by short periods of observation and relatively small increases in prevalence, both of which may not reflect long-term trends.

We and other investigators have shown that the majority of isolates of *M. tuberculosis* in the Western Cape region of South Africa belong to 2 broad lineages: W-Beijing and Euro-American [9–11]. The burden of tuberculosis in this region is among the highest in the world (1037 incident cases per 100,000 individuals in 2005) [12]. Although rates of tuberculosis have historically been high, during the past 4 years, there has been a rapid further increase that has been associated with a concurrent epidemic of HIV infection (figure 1).

We observed an increase in the proportion of W-Beijing strains isolated from children who presented to a major regional pediatric referral hospital over a period of 4 years. To determine whether this reflected a long-term trend, we genotyped strains of *M. tuberculosis* that

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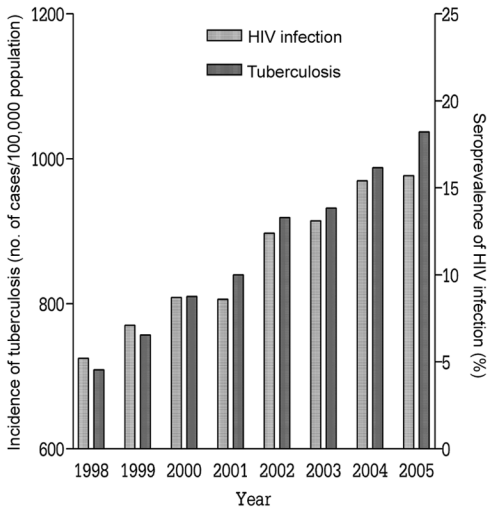

Figure 1. The incidence of active tuberculosis and the seroprevalence of HIV infection (determined by ELISA) among antenatal clinic attendees in the Western Cape region of South Africa during the period 1998–2005 [12].

were isolated from archived histological samples over a period of 76 years. We found that W-Beijing strains have recently emerged and are rapidly becoming more prevalent in Cape Town, which suggests that these strains possess a significant selective advantage.

MATERIALS AND METHODS

Sample Selection

Culture isolates from Red Cross Children's Hospital. During the period 2000–2003, we performed *M. tuberculosis* cultures for children presenting at our hospital (Red Cross Children's Hospital; Cape Town, South Africa) after referral from peripheral clinics or general practitioners. Samples for culture were obtained from respiratory sites (e.g., gastric lavage or induced sputum samples) or, when appropriate, from other disease sites (e.g., CSF, joint aspirate, or lymph node aspirate samples).

Previous studies at our hospital have documented culture confirmation of *M. tuberculosis* in 25% of the children investigated for tuberculosis [13]. Culture-confirmed cases represent a minority of the total number of pediatric cases (~2500 pediatric cases were registered in Cape Town annually during 2000–2002) [14], because specimen collection from children usually requires hospitalization. Therefore, although this sample may represent cases of more severe disease, it was limited by the need for hospitalization to obtain culture specimens.

Archived histological specimens. We obtained a stratified selection of archived postmortem tissue samples from each decade over a 76-year period (1930–2005) from patients who died of active tuberculosis at Groote Schuur Hospital. This hospital is 1 of 2 major tertiary referral hospitals that serve the greater Cape Town region and receives referrals from the same region as Red Cross Children's Hospital. From 1995 through 2005, all tissue samples that we used tested negative for HIV at the time of postmortem examination (a full postmortem examination was not performed for HIV-infected patients). Before 1995, HIV testing was not performed. Demographic details were recorded for each patient. Tissue samples were obtained primarily from the lung, and extrapulmonary sites were selected if affected lung tissue was not available. Hematoxylin-eosin–stained slides were reviewed to confirm evidence of *M. tuberculosis* infection. Samples for which histological characteristics were not supportive of the diagnosis of tuberculosis were excluded.

DNA Extraction

Extraction of mycobacterial DNA from paraffin-embedded tissue samples was performed by using a method based on Chelex100 (Bio-Rad) [15].

Spoligotyping

Spoligotyping was performed as described elsewhere [16]. W-Beijing strains were identified by an easily recognizable pattern (spacers 1–34 were absent, and spacers 35–43 were present). We classified results according to the following criteria: possible W-Beijing strains were defined by the absence of spacers 1–34 and the presence of at least 1 spacer in the region 35–43; W-Beijing strains were defined by the absence of spacers 1–34 and the presence of spacers 35–43; Euro-American strains [9] were defined by the absence of spacers 33–36 and the presence of flanking spacers around this region; other non-Beijing strains were defined by the clear presence of multiple spacers between 1 and 34 (i.e., not W-Beijing), where the deletion of spacers 33–36 was not clearly defined; and unsuccessful was defined as insufficient or no spacers present to identify the strain. We chose the broad Euro-American designation rather than assign these strains a specific classification according to the spoligotype database, because patterns were often incomplete and would not allow specific subclassification.

Quality Control Measures

To prevent cross-contamination, a negative control tissue section was cut from between samples to obtain a "PCR-clean" surface. The microtome blade was replaced and gloves were discarded after each sample was obtained, and the work area was wiped with 10% bleach. DNA extraction and post-PCR procedures were conducted in separate laboratories on separate days. Positive and negative tissue control samples were included during DNA extraction and genotyping.

A successful extraction was defined by an interpretable result in addition to successful spoligotyping of a positive control tissue sample and a negative result for an extraction batch

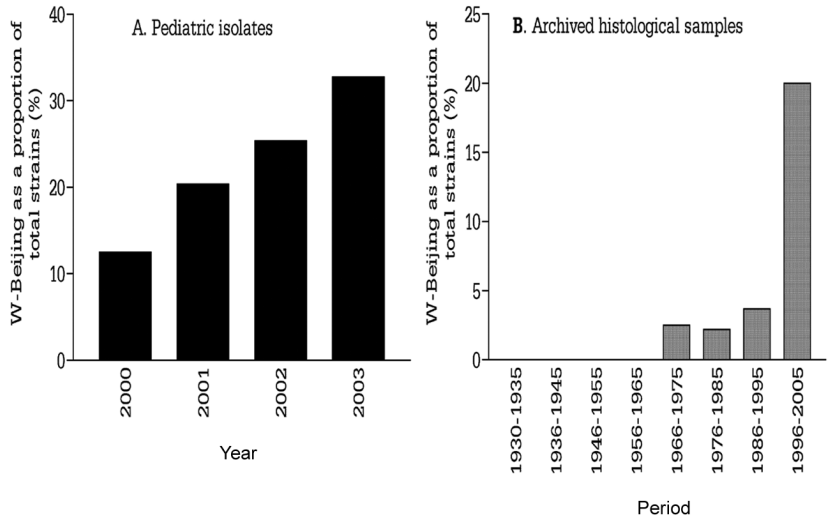

Figure 2. Changes in the proportion of total *Mycobacterium tuberculosis* strains that were W-Beijing strains in Cape Town, South Africa. *A,* The proportion of all isolates from children with culture-confirmed tuberculosis at Red Cross Children's Hospital (Cape Town) from the period 2000–2003 that were W-Beijing isolates ($P = .026$, by Fisher's exact test for 2000–2001 vs. 2002–2003). *B,* The proportion of total *M. tuberculosis*–positive samples found in postmortem tissue samples with histological evidence of tuberculosis from the period 1930–2005 that were positive for the W-Beijing strain (OR for 1930–1975 vs. 1976–2005, 19.05; 95% CI, 2.45–148.3).

control sample (a suspension of Chelex100 with no tissue sample) and a negative control tissue sample. To obtain an interpretable result, it was frequently necessary to repeat the DNA extraction and spoligotyping on independently extracted tissue samples.

Single Nucleotide Polymorphism Analysis

To confirm spoligotype analysis, we amplified and sequenced a 91–base pair fragment of *M. tuberculosis* DNA to detect the presence of a discriminatory single nucleotide polymorphism at position 4280708 (H37Rv) in all samples containing W-Beijing strains and in a random selection of 56 samples containing non–W-Beijing strains. W-Beijing strains contain an adenine, and non–W-Beijing strains contain a guanine base at this position [17]. The following primer pair was used: forward CCTTGGTCGGGCACATTC (H37Rv 4280682–4280699); reverse TAGCGCAGAATCTCTAGGACC (4280772–4280752). The product was purified, and cycle sequencing was performed (for the reverse primer only).

Statistical Analysis

Statistical analysis was performed using Prism Software, version 4.0 (Graphpad Software), and Stata software, version 9 (StataCorp). Nonparametric unpaired data were analyzed using the Mann-Whitney *U* test. Fisher's exact test of probability was used for contingency analyses. The χ^2 test for trend was used to evaluate the changes in ethnicity, age, and strain distribution over time. The Kruskal-Wallis test was used for comparing median ages. We used logistic regression to control for the effects of age and ethnicity on the association between time (measured as a dichotomous variable, either by decade or per designated time period) and the number of W-Beijing strains identified. Statistical significance was inferred by $P < .05$. Approval for this study was obtained from the University of Cape Town Research Ethics Committee (reference 320/2005).

RESULTS

Recent pediatric strains. During 2000–2003, isolates from 291 children were obtained; 68 (23.4%) of these isolates were W-Beijing. The proportion of total strains that were W-Beijing strains increased from 12.5% in 2000 to 32.8% in 2003 ($P = .026$, for 2000–2001 vs. 2002–2003) (figure 2*A*).

The majority of specimens (59%) were of pulmonary origin (30% were gastric lavage specimens, and 29% were induced sputum specimens). W-Beijing strains were no more likely than non–W-Beijing strains to be isolated from extrapulmonary specimens than from pulmonary specimens (risk ratio, 0.91; 95% CI, 0.65–1.27).

The clinical and microbiological features of the pediatric cases are detailed in table 1. HIV infection was confirmed in 19% of children; however, there was no difference between children infected with W-Beijing and those infected with non–W-Beijing strains with respect to the proportion of cases of disseminated disease (12 of 68 children vs. 32 of 223 children; risk ratio, 1.23; 95% CI, 0.67–2.25) or HIV coinfection (15 of 68 children vs. 39 of 223 children; risk ratio, 1.26; 95% CI, 0.74–2.14). The majority of children (267 of 291) lived within

Table 1. Strains of tuberculosis that caused infection at Red Cross Children's Hospital (Cape Town, South Africa) during the period 2000–2003, by associated clinical features.

Strain family	No. of isolates	No. (%) of HIV-positive isolates,[a] %	Site of disease, no. (%) of isolates			Culture specimen				Drug resistance		
			Pulmonary only	Local extrapulmonary	Disseminated	CSF	Gastric washing	Induced sputum	Other extrapulmonary	INH only	RIF only	MDR
X	30	6 (20)	11 (37)	12 (40)	7 (23)	4	5	11	10	2	1	1
W-Beijing	68	15 (22)	33 (48)	23 (34)	12 (18)	9	20	22	17	5	0	0
S	21	2 (9)	9 (43)	8 (38)	4 (19)	5	9	4	3	4	0	0
LAM3	86	13 (15)	38 (44)	37 (43)	11 (13)	8	31	23	24	3	0	0
Other	86	18 (21)	37 (43)	39 (45)	10 (12)	12	23	23	28	6	1	4
Total	291	54 (19)	128	119	44	38	88	83	82	20	2	5

NOTE. Strain families were classified in accordance with the Fourth International Spoligotyping Database [18]. INH, isoniazid; MDR, multidrug resistant (resistant to both INH and rifampicin [RIF]).

[a] Determined by HIV ELISA (with confirmatory PCR for patients aged <1 year); testing was performed only when HIV infection was clinically indicated.

the greater Cape Town metropolitan region. The proportion of children from outside Cape Town did not change over the study period (data not shown).

Strains in archived histological samples. We were able to obtain an interpretable spoligotype pattern in 352 (74.7%) of the 471 selected tissue samples, and in the remainder of tissue samples, the DNA was degraded. Until 1966, none of the 169 identified *M. tuberculosis* patterns were W-Beijing (table 2). Over the next 3 decades, 4 (2.8%) of 138 patterns were W-Beijing, and in the most recent decade (1996–2005), W-Beijing strains were identified in 9 (20%) of 45 tissue samples (OR for 1930–1975 vs. 1976–2005, 19.05; 95% CI, 2.45–148.3) (figure 2*B*). During the most recent decade (1996–2005), all of the W-Beijing strains were identified in samples from the period 2001–2005 (9 of 17 strains), and none were identified in samples from the period 1996–2000. By comparison, there was a trend for the proportion of Euro-American strains to decrease over the study period (OR for 1930–1975 vs. 1976–2005, 0.79; 95% CI, 0.48–1.3).

There were no strains classified as possible W-Beijing (i.e., all strains with spacers 1–34 absent had spacers 35–43 present). However, to confirm the spoligotype classification, we performed single nucleotide polymorphism analysis. We obtained an interpretable sequence from 11 of the 13 W-Beijing tissue samples tested and from 49 of the 56 non–W-Beijing tissue samples tested. In all cases, the sequence data concurred with the spoligotype-assigned classification.

The demographic characteristics of the patients are shown in table 3. The median age (33 years; range, 0.3–93 years) was lower during the earlier decades (1930–1975) than during the later decades (1976–2005; $P < .001$, by χ^2 test for trend), primarily because of the large number of postmortem examinations performed for children with tuberculous meningitis during the earlier decades. The emergence of W-Beijing strains remained statistically significant when all cases of meningitis were excluded (OR for 1930–1975 vs. 1976–2005, 14.04; 95% CI, 1.79–110.5).

We observed a change in the ethnicity of patients, with an increase in the number of black Africans during the more-recent time periods ($P < .001$; by χ^2 test for trend). However black African patients were no more likely than Cape Coloured patients to have been infected with a W-Beijing strain (OR, 1.57; 95% CI, 0.52–4.75). Moreover, after use of logistic regression to control for the effects of age and ethnicity, time remained a significant predictor of the emergence of W-Beijing strains when comparing the period 1930–1975 with the period 1976–2005 (OR, 16.54; 95% CI, 1.90–144.0) or as expressed as an increase in odds per decade (OR per decade, 3.08; 95% CI, 1.58–5.98). Age and ethnicity were not predictors of W-Beijing genotype.

DISCUSSION

Because *M. tuberculosis* is slow growing, rarely undergoes horizontal genetic exchange, and most frequently gives rise to inapparent infections, rapid changes in the population structure of *M. tuberculosis* within regions of endemicity are counterintuitive. However, here we present 2 independent lines of evidence that suggest that W-Beijing strains of *M. tuberculosis* have recently emerged in Cape Town and are rapidly increasing in prevalence.

We initially observed a short-term increase in the proportion of W-Beijing strains among children who received a diagnosis of tuberculosis in Cape Town. Our conclusions from this observation are limited by the short period of study and the fact that cultured samples represent a small proportion of children with tuberculosis in the region (and, thus, may not be fully representative). However, the proportion of W-Beijing strains in 2003 coincides precisely with that observed independently at another pediatric referral center in Cape Town [10]. Because childhood tuberculosis is almost always attributable to progressive primary disease, this proportion is likely to reflect current transmission of W-Beijing strains, as supported by recent studies involving adults [19].

Table 2. Strain lineages (as determined by spoligotyping) of archived histological samples from patients who died of tuberculosis during the period 1930–2005.

Period	No. of samples	Strain lineage, no. (%) of samples		
		W-Beijing	Euro-American	Non-Beijing other
1930–1935	31	0 (0)	28 (90.3)	3 (9.7)
1936–1945	47	0 (0)	39 (83.0)	8 (17.0)
1946–1955	47	0 (0)	39 (83.0)	8 (17.0)
1956–1965	44	0 (0)	34 (77.3)	10 (22.7)
1966–1975	40	1 (2.5)	30 (75.0)	9 (22.5)
1976–1985	44	1 (2.3)	33 (75.0)	10 (22.7)
1986–1995	54	2 (3.7)	38 (70.4)	14 (25.9)
1996–2005	45	9[a] (20.0)	33 (73.3)	3 (6.7)
All	352	13 (3.7)	274 (77.8)	65 (18.5)

[a] By χ^2 comparison of W-Beijing strains in samples from the period 1930–1975 vs. samples from the period 1976–2005 (OR, 19.05; 95% CI, 2.45–148.3).

We confirmed and extended our observations by genotyping strains of *M. tuberculosis* in archived histological postmortem specimens from over a 76-year period. W-Beijing strains were rare or absent in postmortem specimens from Cape Town from 1930 through the mid-1960s. It was only during the most recent decade (1996–2005) that these strains emerged as a major cause of death due to tuberculosis. Although the total number of W-Beijing strains identified was small, the high proportion of these strains identified during the later decades (1976–2005) is supported by the high proportion of W-Beijing strains among our pediatric samples and by findings from studies involving adults [19]. In contrast with our recent pediatric data, our evaluation of strains present in archived histological material did not identify a single W-Beijing strain in samples from 116 children (age, <10 years) during the period 1930–1965.

We elected to use spoligotyping as the primary method for identifying W-Beijing strains in the postmortem specimens, because spoligotyping is regarded as highly accurate for defining this lineage [20] and is suitable for use on degraded DNA. Because DNA in paraffin-embedded tissues is variably degraded [21], it is not possible to use other classic genotyping methods on these tissue specimens.

There is recent evidence of a group of "ancestral" W-Beijing strains that do not show the characteristic deletion of spacers 1–34 but share a common ancestry with "modern" W-Beijing strains [9]. However, because there were no "ancestral" W-Beijing strains in our pediatric collection, we did not attempt to identify these strains in the archived histological material.

We previously performed multiple interspersed repetitive unit typing of the pediatric strains reported here [11], and we found that the population structure of "modern" W-Beijing strains in the Western Cape is heterogeneous, including at least 2 major lineages, with considerable diversity within each lineage. The emergence of W-Beijing strains in Cape Town is, therefore, not attributable to a clonal outbreak of a single strain, but it is, rather, attributable to the expansion of several sublineages. Our findings differ from those of previous reports of clonal outbreaks of W-Beijing strains with respect to this important phenomenon [22, 23].

Potential confounders in the analysis of the strains from histological material include changes in age and ethnicity over the period studied. The younger age of patients undergoing postmortem examinations during the earlier years (1930–1955), compared with the age of patients during the later years (1956–2005) was attributable to a large number of postmortem examinations performed for children with tuberculous meningitis. It may be argued that certain strains may have a predilection for meningeal disease. However, when we excluded all samples from patients with meningitis, W-Beijing strains were still strongly overrepresented during the later decades (1976–2005). In Vietnam, Anh et al. [24] revealed that the W-Beijing genotype was associated with young age, suggesting that W-Beijing strains may be emerging. In our postmortem samples, age was not a predictor of W-Beijing genotype, but this may reflect the small number of samples from children during more-recent decades (this would tend to underestimate the extent of the emergence of W-Beijing strains).

There was a change in the ethnicity of patients who underwent postmortem examinations during the period studied, probably as a result of urban migration of individuals of black African origin to Cape Town [25]. This change long preceded the emergence of W-Beijing strains and is unlikely to account for our observation of emergence. Moreover, there was no association between ethnicity and strain lineage by multivariate analysis. International migration is unlikely to account for our findings, because there has been no documented significant

Table 3. Demographic characteristics of patients from whom postmortem samples were available for strain genotyping.

Period	No. of patients	Age, median years (range)	Female sex, %	Ethnicity, no. (%) of patients			
				Black African	Cape Coloured[a]	European	Unknown
1930–1935	45	3.0 (0.3–55.0)	57.8	5 (11.1)	32 (71.1)	7 (15.6)	1 (2.2)
1936–1945	78	11.0 (0.3–69.0)	46.2	10 (12.8)	51 (65.4)	15 (19.2)	2 (2.6)
1946–1955	65	5.0 (0.3–69.0)	41.6	16 (24.6)	34 (52.3)	13 (20.0)	2 (3.1)
1956–1965	55	33.0 (0.3–93.0)	49.1	23 (41.8)	27 (49.1)	4 (7.3)	1 (1.8)
1966–1975	52	40.5 (0.4–75.0)	42.4	25 (48.1)	24 (46.2)	3 (5.8)	0 (0)
1976–1985	56	44.5 (0.8–77.0)	48.3	29 (51.8)	20 (35.7)	7 (12.5)	0 (0)
1986–1995	71	52.0 (2.5–84.0)	43.7	42 (59.1)	28[b](39.4)	1 (1.4)	0 (0)
1996–2005	49	43.0 (18.0–82.0)	51.0	28 (57.1)	20 (40.8)	0 (0)	0 (0)

[a] See the Discussion section for an explanation of this population group [27].
[b] Includes 1 Asian patient.

migration from Eastern Asia, Russia, or eastern Europe (where W-Beijing is prevalent) to the Cape Town region in recent years.

Before European settlement of the Western Cape region 400–500 years ago, the population density of indigenous Khoi-San peoples was low, and historical records suggest that tuberculosis was rare or unknown [26]. European settlement is believed to have introduced the disease; indeed for a few years, the Cape was promoted as a health resort for European consumptives [26]. This coincides with our finding that the majority of strains found during the early years of the 20th century belong to the Euro-American lineage, which predominates in Europe. Admixture between East Asian, Khoi-San, European, and black African populations gave rise to the so-called Cape Coloured population of the province [27]. Because W-Beijing strains are common in East Asia, it was surprising that W-Beijing strains were uncommon (or absent) during the early decades of this study (when the majority of tissues were from patients of Cape Coloured origin). W-Beijing strains are now common among Cape Coloured patients with tuberculosis [11, 19, 28].

What are the potential reasons for the emergence of these strains? Their rapid expansion (as a proportion of total strains) suggests that factors other than a general failure in tuberculosis control may be important. The association between W-Beijing strains and drug resistance has been reported in Cape Town [10] and elsewhere [29–31]; however, the majority of W-Beijing strains in Cape Town remain susceptible to drugs [10] (93% of strains were susceptible to isoniazid and rifampicin in our pediatric cases) (table 1).

Caws et al. [4] documented a strong association between the W-Beijing lineage and HIV infection in Vietnam. An outbreak of infection due to the W-Beijing strain in New York City primarily occurred among HIV-infected persons (prevalence, 86%) [32]. It is, therefore, interesting that the emergence of the W-Beijing strain coincided with the spread of HIV infection in the Western Cape. However, because the tissue samples used for this study originated from HIV-uninfected persons, it is not possible to determine whether HIV infection has contributed to the emergence. Because HIV coinfection is associated with a high rate of progression to active disease [33], rapid changes in strain distribution may be more likely to occur in a population with a high prevalence of HIV infection.

The increased virulence of some W-Beijing strains in experimental models has been related to subversion of innate immune responses by the production of phenolic glycolipid [34] or by the induction of IL-10–producing regulatory T cells [35]. W-Beijing strains have also been shown to have constitutive up-regulation of the DosR dormancy regulon [36], which may confer a survival advantage in anaerobic conditions. It is feasible (although unproven) that immune subversion may result in a high proportion of infections progressing to active disease.

Immune subversion may also be related to an increased bacillary load, as demonstrated in some animal models [6]. HIV coinfection, which has been associated with increased bacillary burden [37], may have a synergistic effect. An increased bacterial load in the lung may increase transmissibility and may also increase the likelihood of dissemination to extrapulmonary sites, which is reflected in the association between the W-Beijing strain and extrapulmonary tuberculosis [3].

Some authors have suggested that bacille Calmette-Guérin vaccination might feasibly select for the spread of W-Beijing strains [7, 38, 39]. Routine bacille Calmette-Guérin vaccination in South Africa commenced in 1973 and is now almost universal [12], overlapping closely with the period over which W-Beijing strains have expanded. We do not, however, have any data specifically linking W-Beijing strains with prior bacille Calmette-Guérin vaccination.

To our knowledge, these are the first data to clearly document the emergence and rapid spread of W-Beijing strains of tuberculosis in a region where tuberculosis is endemic over an ex-

tended period. Our findings suggest that these strains possess a significant advantage in their ability to disseminate within a community. The biological basis for this advantage needs to be elucidated to understand and intervene in the spread of these strains.

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