

Association of *Mycobacterium tuberculosis* complex isolates of BOVIS and Central Asian (CAS) genotypic lineages with extrapulmonary disease

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Abstract

The association between isolate genotype, defined as in the international spoligotype database SpolDB4, and extrapulmonary tuberculosis was determined among 1009 patients in a population-based, 4-year survey performed in Tuscany, Italy. Extrapulmonary disease occurred in 24.2% of patients. A statistically significant association with extrapulmonary disease was found for the BOVIS (adjusted OR 3.2; 95% CI 1.2–8.1) and for the Central Asian (CAS) lineages (adjusted OR 2.3; 95% CI 1.0–5.1). These findings support the view that *Mycobacterium tuberculosis* strains within individual genotypic lineages might have evolved unique pathogenic characteristics that are capable of influencing the clinical outcome of the infection.

Keywords: Extrapulmonary tuberculosis, genotyping, *Mycobacterium tuberculosis*, SpolDB4, spoligotyping

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Introduction

In the past, much tuberculosis (TB) research was based on the assumption that TB is caused by a highly conserved strain of *Mycobacterium tuberculosis*. However, recent studies on the genomics and molecular evolution of *M. tuberculosis* have shown that the organism has evolved in large, geographically constrained, clonal lineages, without horizontal genetic exchanges, thus yielding a large clonal population of genotypically and phenotypically different lineages, potentially endowed with different properties that can impact on the virulence of the organism and the consequent disease [1,2]. Although it has long been known, mainly through animal studies, that certain strains show increased virulence [2], there is little compelling evidence that specific genetic lineages show peculiar pathogenic properties in humans. The possible exception concerns the strains of the Beijing genetic lineage, which, in certain settings, have been shown to be capable of spreading rapidly

in large clonal clusters [3], and have been linked with extrapulmonary TB or with treatment failure and relapse [4–7]; in animal studies, Beijing strains induced a more severe pathology than other *M. tuberculosis* genotypes, and elicited a distinct immune response with a reduced cytokine response, resulting in a failure to induce Th1-type immunity [2,8,9].

The ability of a strain to disseminate and cause extrapulmonary TB is considered a marker of virulence. In fact, the increased infectivity of strains that are found at extrapulmonary locations has been demonstrated by the studies of Garcia de Viedma *et al.* [10]. In the present study, the association of extrapulmonary TB with the *M. tuberculosis* genotypic lineages defined in the SpolDB4 database [11] using the spacer oligonucleotide typing assay (spoligotyping) [12] was determined in a population-based, 4-year survey performed in Tuscany, Italy, a region with a low prevalence of TB, where different autochthonous and imported *M. tuberculosis* genotypic lineages coexist, owing to a recent increase in immigration from high-prevalence countries [13].

Materials and Methods

Clinical isolates and patients

A total of 1009 *M. tuberculosis* complex strains, collected by the Regional Reference Centre for Mycobacteria, Florence,

Italy, during a 4-year study period from 1 January 2002 to 31 December 2005 from the same number of TB patients (563 Italian-born; 446 foreign-born), admitted to ten major community hospitals in Tuscany, Italy, were studied. The study isolates represent the majority (>90%) of the total isolates in the region during the study period. All isolates were identified by DNA probes (Accuprobe (Gen-Probe, San Diego, CA, USA) and/or InnoLipa (Innogenetics, Gent, Belgium)). *Mycobacterium bovis* BCG isolates were not included in the present study. The patients' clinical records were reviewed in order to classify the disease as pulmonary or extrapulmonary. Twelve patients with concomitant pulmonary and extrapulmonary disease were placed in the extrapulmonary disease group (Table 1).

Spoligotyping

Spoligotyping analysis of isolates was performed as described by Kamerbeek *et al.* [12]. Briefly, genomic mycobacterial DNA was extracted from the bacteria grown on ADC-supplemented Middlebrook 7H9 or Lowenstein-Jensen medium (Becton Dickinson, Baltimore, MD, USA) by the cetyltrimethylammonium bromide method. The oligonucleotides DRa and DRb were used as primers to amplify the whole direct repeat (DR) region by PCR. The amplified biotinylated products were hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed, incubated in streptavidin-peroxidase conjugate (Boehringer, Mannheim, Germany), and finally

developed by chemoluminescence. *M. tuberculosis* complex isolates were assigned to one of the genotypes (or families) defined in the international spoligotype database SpolDB4 of the Pasteur Institute of Guadeloupe, France [11]. The currently accepted nomenclature of the genotypic lineages of SpolDB4 was also retained, to refer to clades BOVIS and AF-RICANUM, given in capital letters, which essentially include the species *M. bovis* and *Mycobacterium africanum* of the *M. tuberculosis* complex.

Statistical analysis

The associations of extrapulmonary TB with the *M. tuberculosis* genotypes were assessed by univariate chi-square test and multiple logistic regression. In the multiple logistic regression, each genotype was included in the model as a dummy variable by using the T genotype as a reference category. This approach allowed assessment of the relative importance of each genotype in predicting the probability of extrapulmonary localization. The T genotype was used as a referent because it was the largest genotype and showed a proportion of pulmonary and extrapulmonary TB comparable to that of the overall study population (Table 1). Analysis was performed by SAS software, version 8.2 (SAS Institute, Cary, NC, USA).

Results and Discussion

A total of 1009 strains of *M. tuberculosis* complex, isolated during a 4-year period in Tuscany, Italy, were genotyped by

TABLE 1. Distribution of pulmonary and extrapulmonary tuberculosis cases classified according to major *Mycobacterium tuberculosis* genotypic lineages during the period 2002–2005 in Tuscany, Italy

Genotype	No. of isolates (%)			Association of genotypes with extrapulmonary disease ^a				
				Univariate χ^2		Multiple logistic regression		
	Pulmonary	Extrapulmonary ^b	Total	χ^2	p	Adjusted OR	95% CI	p
Africanum	8 (57.1)	6 (42.9)	14	2.65	NS	2.139	0.718–6.368	NS
Beijing	60 (82.2)	13 (17.8)	73	1.82	NS	0.618	0.321–1.190	NS
Bovis	8 (47.1)	9 (52.9)	17	8.43	0.004	3.168	1.239–8.100	0.016
CAS	15 (55.6)	12 (44.4)	27	6.10	0.014	2.281	1.021–5.097	0.044
EAI	35 (77.8)	10 (22.2)	45	0.114	NS	0.815	0.384–1.726	NS
Haarlem	178 (82.4)	38 (17.6)	216	6.780	0.009	0.609	0.392–0.944	0.027
LAM	98 (76.0)	31 (24.0)	129	0.007	NS	0.902	0.556–1.462	NS
S	44 (84.6)	8 (15.4)	52	2.383	NS	0.518	0.233–1.152	NS
T	211 (74.0)	74 (26.0)	285	0.577	NS	1.000	–	–
X	10 (83.3)	2 (16.7)	12	0.387	NS	0.570	0.122–2.663	NS
Others ^c	4 (100.0)	0 (0.0)	4	–	–	–	–	–
Undefined ^d	94 (69.6)	41 (30.4)	135	3.090	NS	1.244	0.791–1.955	NS
Total	765 (75.8)	244 (24.2)	1009	–	–	–	–	–

NS, not significant.

^aAssessed by univariate chi-square test and multiple logistic regression model using the T genotype as a referent.

^bThis group includes a total of 12 isolates with concomitant pulmonary and extrapulmonary localization (four of T genotype; three of Haarlem genotype; two of CAS genotype; two of Beijing genotype; one of undefined genotype).

^cThis group contains two isolates with a signature attributable to the Haarlem or S family, one isolate with a signature attributable to the Haarlem or T family, and one isolate of MANU family.

^dStrains with a signature that is as yet undefined in SpolDB4.

spoligotyping and assigned to one of the clades defined in the international spoligotype database SpolDB4 of the Pasteur Institute of Guadeloupe, France [11]. Spoligotyping identified ten major genotypic families, namely, AFRICANUM (1.4% of total isolates), Beijing (7.2%), BOVIS (1.7%), Central Asia (CAS) (2.7%), East Africa–India (EAI) (4.5%), Haarlem (21.4%), Latin American–Mediterranean (LAM) (12.8%), S (5.2%), T (28.2%), X (1.2%), and other spoligotypes undefined in SpolDB4 (13.8%). The distribution of pulmonary and extrapulmonary TB among the genotype families is reported in Table 1. As shown, the extrapulmonary disease accounted for 244 cases (24.2%); extrapulmonary TB cases ranged from 15% for isolates of the S genotype to 53% for isolates of the BOVIS genotype. Using the T genotype as a referent, both univariate chi-square test and multiple logistic regression indicated that extrapulmonary TB is significantly associated with infection by isolates of the BOVIS genotype (adjusted OR 3.2; 95% CI 1.2–8.1) and isolates of the CAS genotype (adjusted OR 2.3; 95% CI 1.0–5.0). For the AFRICANUM genotype, the percentage of extrapulmonary TB cases was as high as 43%, but it did not reach statistical significance, owing to the small numbers.

The epidemiological features of patients with TB caused by the BOVIS and CAS genotypes are reported in Table 2. Most patients with TB caused by the BOVIS genotype (11/17) were Italian-born, and ranged from 33 to 93 years of age (mean, 69 years), which probably indicates, at least in most cases, a reactivation of an *M. bovis* infection acquired long before; according to clinical records, there was a previous TB episode for only one Italian-born, 74-year-old patient. The six foreign-born patients with BOVIS TB were young (mean age, 28 years; range, 21–38 years), thus indicating a recently acquired infection. All BOVIS isolates were

susceptible to rifampin; nine/14 tested isolates (64%) were resistant to pirazinamide; two isolates were resistant to isoniazid; and two isolates were resistant to ethambutol. No association between drug resistance and extrapulmonary TB was observed. On the other hand, all patients with TB caused by the CAS strains were young (mean age, 32 years; range, 20–45 years) and foreign-born, being mostly from South Asia, especially Pakistan and India, and from Africa; according to clinical records, among the 27 patients there was no previous TB episode for 19 patients, and this information was unknown for eight patients. This suggests that the CAS patients acquired infection in their countries of origin and developed the disease after arrival in the host country. In general, all CAS isolates were susceptible to first-line anti-TB drugs, with the exception of two isolates resistant to isoniazid, and two isolates resistant to pirazinamide; no multidrug resistance was observed among the isolates. Human immunodeficiency virus status, when known, was negative for all patients harbouring BOVIS or CAS genotypes, thus ruling out human immunodeficiency virus infection as a factor influencing the extrapulmonary localization of TB for both genotypes.

The molecular characteristics of the BOVIS and CAS isolates are shown in Table 3. A total of seven different spoligotype shared types (an identical pattern shared by two or more patients, referred to as Spoligotype International Types (SITs)) were detected for the BOVIS genotype; SIT 482 (BOVIS1_BCG clade) was prevalent. Typing of BOVIS isolates by the potentially highly discriminatory variable-number tandem repeat–mycobacterial interspersed repetitive units (VNTR-MIRU) assay [14] was not performed, because, unlike for *M. tuberculosis* isolates, whose VNTR-MIRU profile is generally considered to be an individual marker equivalent to

TABLE 2. Epidemiological features of patients with pulmonary and extrapulmonary tuberculosis caused by *Mycobacterium tuberculosis* of the BOVIS or CAS genotypic lineages

Patients	Lineage			
	BOVIS		CAS	
	Pulmonary	Extrapulmonary	Pulmonary	Extrapulmonary
Male/female ratio	2.5	0.3	3.7	3.0
Age ^a	60 (21–80)	51 (24–93)	33 (24–45)	31 (20–44)
Country of birth ^b	Italy (6) Morocco (1) Peru (1)	Morocco (2) Philippines (2)	Pakistan (11) India (1) Iraq (1) Ivory Coast (1) Unknown (1)	Pakistan (6) India (2) Somalia (2) Cameroun (1) Unknown (1)
HIV status ^b	Negative (3) Unknown (5)	Negative (8) Unknown (1)	Negative (14) Unknown (1)	Negative (9) Unknown (3)

HIV, human immunodeficiency virus.

^aExpressed as mean (the range is given in parentheses).

^bThe number of tuberculosis patients is given in parentheses.

involved foreign-born individuals, the possible delay in diagnosis and treatment of TB infection in these patients may have caused the progression of primary TB infection to extrapulmonary sites.

In the present survey, a low percentage (18%) of extrapulmonary localization was observed among cases of Beijing TB; similarly, a study performed in South Africa also failed to demonstrate an association between Beijing strains and the propensity to cause extrapulmonary disease [32]. These data contrast with those of Kong et al. [5], who reported that patients infected with the Beijing strains were nearly three times as likely as patients infected with the non-Beijing strains to have extrathoracic involvement, suggesting that the Beijing strains may have some special biological features that facilitate the development of extrathoracic TB. However, other studies have indicated that only certain sublineages of the Beijing family show peculiar pathogenic features; for example, extrathoracic Beijing TB was found to be associated only with sublineages bearing specific genomic deletions in one study [4], and in another study, only a recently evolved Beijing sublineage showed an increased ability to be transmitted (and to cause disease). Nonetheless, an association between the sublineage and clinical parameters could not be demonstrated [33].

In conclusion, the present data lend support to the view that the pathogenic properties of *M. tuberculosis* are not evenly conserved among the genotypic lineages of the *M. tuberculosis* complex, and that strains within individual lineages or sublineages might have evolved unique pathogenic characteristics that influence the clinical outcome of the infection.

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Transparency Declaration

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