

## First Molecular Epidemiology Study of *Mycobacterium tuberculosis* in Burkina Faso<sup>∇</sup>

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We conducted a molecular epidemiology study on 120 *Mycobacterium tuberculosis* isolates from patients presenting pulmonary tuberculosis (TB) in Burkina Faso. Classical antibiogram studies and genetic characterization, using mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) typing and spoligotyping, were applied after culture. Molecular analysis of specific signatures showed that all TB cases reported in this study were caused by *M. tuberculosis* and identified no *Mycobacterium bovis* or *Mycobacterium africanum* isolates. This result is unexpected, as *M. africanum* strains were reportedly the etiologic agent in 20% of TB cases 2 decades ago. The comparison of spoligotypes from Burkina Faso with an international spoligotype database (SpolDB4) showed that the majority of isolates belong to major clades of *M. tuberculosis* (Haarlem, 9%; Latin American-Mediterranean, 30%; and T, 20%). The predominant group of isolates (30%) corresponds to spoligotype 61, described in Cameroon as the “Cameroon family.” In Burkina Faso, as in Cameroon, this family could be associated with recent transmission of TB, suggesting a recent expansion in West Africa. Our data suggest a low level of primary drug resistance that may be a positive result of the Directly Observed Therapy Shortcourse program. Besides, based on spoligotyping plus MIRU-VNTR, data showed a high number of clusters in our sample, suggesting a high level of recent TB transmission in Burkina Faso. Nevertheless, an important genetic polymorphism was observed in this country, reflecting an endemicity situation where the control of TB would have less impact in the main towns.

Every year nearly 9 million people contract tuberculosis (TB) and close to 2 million die from the disease (34). The success of propagation of this disease remains directly linked to the social and hygiene conditions of human populations. Nevertheless, the emergence of multidrug-resistant strains (defined as resistance to at least isoniazid and rifampin) and the high incidence of human immunodeficiency virus (HIV) have strongly contributed to the reemergence of TB in many parts of the world (34).

In Africa, the incidence rate has been increasing since 1990 both in countries with low HIV rates and in countries with high HIV rates (34). In 2003, the estimated number of new TB cases on this continent exceeded 2 million, with almost 600,000 deaths (34). Furthermore, according to WHO, HIV prevalence in TB cases goes beyond 50% in South Africa and 30 to 40% of TB patients who die present a TB-HIV coinfection (34).

Burkina Faso is a West African country with a total population of over 13 million people. As in numerous other African countries, TB is a major health scourge, in conjunction with AIDS, malaria, and various other parasitoses. Between 1990 and 2003, the estimated TB incidence slowly increased from 147 to 163 cases per 100,000 inhabitants (34). In 2003 among the new tuberculosis cases, the estimated HIV seroprevalence and proportion of multidrug-resistant TB cases were 23% and

2.6%, respectively, in the ranges of those of other West African countries (34).

Little epidemiological and molecular data are available on human TB in Burkina Faso, as in most other African countries. Only one strain from Burkina Faso has been described in the worldwide SpolDB4.0 spoligotyping database of a total of 39,295 entries (4). Two studies, published in 1989 and 1996 (17, 22, 25), investigated the distribution of *Mycobacterium tuberculosis* members based only on phenotypic identification, in samples of 102 and 229 TB isolates in two different settings in this country. Both reported a prevalence of 17 to 18% *Mycobacterium africanum* isolates, while the rest of the sample populations were essentially composed of *M. tuberculosis*. Interestingly, another study recently indicated a strong decrease in the prevalence of *M. africanum* isolates among TB cases between the 1970s and late 1990s in Cameroon, another West African country. This observation suggested a marked change in the pathogen population in the region (20).

This paper presents the first molecular epidemiological study of human TB in this country, as a step to a better understanding of TB epidemiology in Africa. For genetic characterization, we used two different typing systems: spoligotyping targeting the direct repeat locus (15) and mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) typing based here on 12 independent loci (19, 28). These two techniques used in combination are now increasingly used as an alternative to standard IS6110-based restriction fragment length polymorphism genotyping for molecular epidemiological studies. These two PCR-based methods are

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less technically demanding and faster. Population-based studies integrating demographic and contact investigation data in the United States demonstrated that using spoligotyping and MIRU-VNTR typing together can provide adequate discrimination for molecular epidemiological analysis in comparison to IS6110 restriction fragment length polymorphism, when applied to regional areas (5).

The specific objectives of this study were to estimate the prevalence of *M. tuberculosis* complex members among recent human TB cases in the two main towns of Burkina Faso, to identify and evaluate the diversity of the *M. tuberculosis* families circulating in this country, and to evaluate the rate of drug resistance and the families associated with drug resistance.

#### MATERIALS AND METHODS

**Patients.** The study was conducted over a 1-year period (January to December 2001). During this period, 120 patients with symptomatic disease and sputum culture positive for *M. tuberculosis* complex were included. All patients were new cases and naïve for TB treatment. One isolate per patient was studied. Forty-one patients came from different centers and hospitals from Ouagadougou, the capital of Burkina Faso, and 79 patients from Bobo-Dioulasso, the second largest city in this country. The different centers and hospitals were the Centre National de Lutte Antituberculeuse (19 patients) and the Centre National Hospitalier de Yalgado Ouedraogo (22 patients) in Ouagadougou and the Centre Régionale de Lutte Antituberculeuse (60 patients) and the Centre National Hospitalier de Sourou Sanou (19 patients), situated in Bobo-Dioulasso. Antibodies to HIV type 1 (HIV-1), HIV-1 group O, and HIV type 2 (HIV-2) were detected by a commercial enzyme immunoassay (Murex HV-1.2.0; Abbott Laboratories, Abbott Park, IL). If this test was reactive, HIV-1 and HIV-2 infections were differentiated by using two commercial monospecific tests, Wellcozyme HIV Recombinant for HIV-1 and Murex HIV-2 for HIV-2 (Abbott Laboratories). All of these analyses were carried out in the Mycobacteriology Laboratory and the Biological Laboratory of the Muraz Centre situated in Bobo-Dioulasso. From these analyses, out of the 120 patients, 43 (35.8%) were HIV-1 positive, with 21 females (48%) and 22 males (52%); 77 (62%) were HIV negative.

**Strain culture, drug susceptibility testing, and *M. tuberculosis* complex identification.** In the Biological Laboratory of the Muraz Centre situated in Bobo-Dioulasso, culture of mycobacteria was done on Löwenstein-Jensen (LJ) slants after treatment of the samples by the Petroff method and the classical biochemical tests for the identification of *M. tuberculosis* complex. One hundred twenty cultures confirmed to be positive by Ziehl-Neelsen staining were transported on LJ egg medium to the Laboratoire de Bactériologie du Centre Hospitalo-Universitaire Arnaud de Villeneuve, Montpellier, France, for further characterization. Subcultures were performed using the radiometric broth method (BACTEC; Becton Dickinson Diagnostic Systems, Sparks, MD) and LJ egg medium to confirm the lack of contamination. All 120 isolates included in this study were identified as belonging to the *M. tuberculosis* complex using the AccuProbe (GenProbe, San Diego, CA) test. A DNA strip assay (Genotype MTBC; Hain Lifescience, Nehren, Germany) was used for the differentiation of the *M. tuberculosis* complex members.

Susceptibilities to isoniazid, rifampin, ethambutol, and streptomycin were tested with the radiometric broth method (BACTEC; Becton Dickinson Diagnostic Systems). The concentrations of the antituberculosis drugs used were as follows: isoniazid, 0.1 g/ml; rifampin, 2.0 g/ml; ethambutol, 2.5 and 7.5 g/ml; and streptomycin, 2.0 and 6.0 g/ml.

**Genotyping.** Genomic DNA was extracted from the primary LJ egg culture as described previously (30). Spoligotyping was performed as previously described by Kamerbeek et al. (15). The data obtained were compared with the international SpolDB4.0 database, containing 35,925 spoligotypes from 39,295 isolates from 122 countries.

MIRU-VNTR loci referred to as MIRU 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 were individually amplified and analyzed as previously described by Supply et al. (28). Results from each of the 12 loci were combined into 12-digit allelic profiles (28).

**Genetic diversity analyses.** To explore the genetic variability of the isolate population studied, the mean genetic diversity (24) was used and calculated according to the formula  $H = \sum h/n$ , where  $h = 1 - \sum q_i^2$ ,  $n$  = number of loci,  $h$  = allelic diversity of each locus, and  $q_i^2$  = relative frequency of the  $i$ th allele for the locus considered. The Hunter-Gaston discriminatory index (HGDI) was

used to estimate the discriminatory power of MIRU-VNTR and spoligotyping. HGDI was calculated as follows:

$$\text{HGDI} = 1 - \left[ \frac{1}{N(N-1)} \sum_{j=1}^s nj(nj-1) \right]$$

where  $N$  is the total number of strains in the typing scheme,  $s$  is the total number of different MIRU-VNTRs and/or spoligotype patterns, and  $n_j$  is the number of strains belonging to the  $j$ th pattern (14).

**Phylogenetic analysis.** Phylogenetic relationships among the isolates were inferred from spoligotyping and MIRU data using different phylogenetic methodologies. For all analyses, a *Mycobacterium canettii* genotype was used as the outgroup (27). First, we used the neighbor-joining analysis (23) to construct a phenetic tree based on Jaccard distance matrices. Second, Wagner analysis (8) with bootstrapping was used to test the robustness of the nodes. The PHYLIP software (version 3.5c; J. Felsenstein, Department of Genetics, University of Washington, Seattle, 1993) and Treedyn software, designed in our laboratory (www.treedyn.org) (4a), were used to analyze the association between the tree topology and properties of the isolates under study, such as the geographical origin, sex, and HIV status.

**Statistical analysis.** Statistical analyses were performed using StatView software, version 4.5 (SAS Institute Inc., Cary, NC). Differences in median continuous variables were calculated using the Mann-Whitney test. Associations between variables were assessed using the  $\chi^2$  test. Logistic regression analyses were performed to determine the differences between groups; odds ratios with 95% confidence intervals were calculated.  $P$  values of <0.05 were considered statistically significant. A strain cluster was defined as two or more patients infected by strains having identical spoligotypes and MIRU-VNTR patterns.

#### RESULTS

**Study population.** Between 1 January and 31 December 2001, 120 cases of pulmonary TB that were culture positive for the *M. tuberculosis* complex were included in the analysis. The age of patients ranged from 15 to 75 years (mean age, 34 years). The female-to-male sex ratio was 2:5, with similar distribution in the two towns. From these analyses, out of the 120 patients, 43 (35.8%) were HIV-1 positive, with 21 females (48%) and 22 males (52%); 77 (62%) were HIV negative.

**Drug susceptibility patterns of strains.** Because of problems with management of the laboratory work, antibiotic susceptibility was tested on the 120 subcultures 6 months after their arrival in Montpellier, France, and we could obtain antibiogram results for only 61 strains out of 120. Fifty-eight out of the 61 (95.1%) strains were sensitive to all four first-line drugs (isoniazid, rifampin, ethambutol, and streptomycin), two (3.3%) strains were resistant to isoniazid, and only one (1.6%) was multidrug resistant (isoniazid and rifampin resistant). Of the three patients infected by drug-resistant strains, one was HIV positive and two HIV negative. All three were male and 28, 34, and 72 years of age, respectively.

**Identification and genotyping of *M. tuberculosis* strains.** Methods of *M. tuberculosis* complex molecular identification assigned all of the 120 isolates to the *M. tuberculosis* complex and to *M. tuberculosis sensu stricto*.

Spoligotyping confirmed *M. tuberculosis* identification for all cases. Thirty-eight patterns were detected among the 120 isolates. A total of 95 (79.2%) isolates were grouped into 13 clusters, whereas 25 (20.8%) presented a single spoligotype (Table 1).

The 38 spoligotypes (STs) were compared with those contained in the international spoligotyping database (SpolDB4) (4). A total of 25 spoligotypes were already described in SpolDB4, while 13 were new and unique (orphan). The largest

TABLE 1. Characteristics of clustered and nonclustered strains determined by spoligotyping plus MIRU-VNTR

Epidemiological characteristic	No. (%) of patients infected by:		P value
	Clustered strain	Nonclustered strain	
Age < 35 yr	37 (49.3)	38 (50.7)	0.25
Male sex	46 (52.9)	41 (47.1)	0.86
HIV-1-seropositive status	27 (62.8)	16 (37.2)	0.10
Geographic origin			
Ouagadougou	42 (53.2)	37 (46.8)	0.95
Bobo-Dioulasso	32 (62.8)	19 (37.2)	0.95

cluster consisted of 36 strains belonging to the Latin American-Mediterranean (LAM) family and exclusively to the so-called LAM10 type corresponding to spoligotype 61 in the international database. Another important cluster (26 strains) corresponded to the ubiquitous T spoligotype, designated in the SpolDB4 database as spoligotype 53. Two other clusters belonged to the ubiquitous Haarlem family, spoligotype 47 (six strains) and spoligotype 50 (five strains). The nine other clusters obtained in this study did not belong to a defined spoligotype family but were designated in the spoligotype database by a number. They corresponded to one cluster of five strains (ST 772), two clusters of three strains (STs 200 and 740), and six clusters of two strains (STs 523, 78, 494, 57, 62, and 848). The spoligotypes of drug resistance strains corresponded to the T spoligotype (two strains) and to ST 740 (one strain), which has been found only six times in The Netherlands, Germany, Austria, Guadeloupe, and Peru in SpolDB4. The spoligotypes found from the strains of the 43 HIV-infected patients are distributed as follows: ST 61 (LAM10) (37.2%), orphan (21%), ST 53 (T) (18.6%), ST 50 (Haarlem) (4.65%), ST 200 (7%), ST 772 (4.65%), ST 740 (2.3%), ST 78 (2.3%), and ST 62 (2.3%).

Seventy-one different MIRU-VNTR patterns were detected among the 120 isolates. They were distributed in 20 clusters comprising 69 strains (57.5%) and 51 unique patterns (42.5%) (Table 1). The largest cluster comprised 15 strains, 10 clusters comprised two strains, six clusters comprised three strains, and two clusters comprised five strains.

The 36 isolates with the same LAM10 spoligotype were divided into 14 different MIRU genotypes. Likewise, the 26 isolates with the same T spoligotype identified by spoligotyping were divided into 16 different MIRU genotypes.

Isolates from three patients showed a combination of double alleles detected in several MIRU-VNTR loci. We could not determine whether these were due to mixed infection or to laboratory contamination; consequently, these samples were excluded from our analysis.

#### Combined analysis of spoligotyping and MIRU-VNTR data.

As a result of the discrimination of a few MIRU-clustered isolates by spoligotyping (see above), the combination of spoligotyping and the MIRU-VNTR typing provided the highest discriminatory power, with 77 distinct patterns, 64 isolates (53.3%) grouped into 21 clusters and 56 isolates (46.7%) with unique patterns. Table 1 summarizes the overall distribution of clustered strains. The three patients infected by resistant strains belonged to three different clusters; the drug susceptibilities of the other isolates of these three clusters were not

available. Among 43 HIV-infected patients, 28 (62.8%) harbored strains included in clusters.

The discriminative power of MIRU-VNTR and spoligotyping was calculated with the HGDI (14). While the HGDI was 86% and 96% for spoligotyping and MIRU-VNTR, respectively, the HGDI increased to 98% for the combination of the two techniques (Table 1). Consistently, the mean genetic diversity ( $H$ ) was significantly lower for spoligotyping data than for MIRU-VNTR with 12 loci (spoligotyping,  $H = 0.15$ , versus MIRU-VNTR,  $H = 0.29$ ;  $P = 0.007$ ).

**Phylogenetic analysis.** The dendrogram (Fig. 1) was generated using the UPGMA (unweighted-pair group method using average linkages) algorithm based on the spoligotype and MIRU-VNTR data detailed in Fig. 1. From the phylogenetic tree, we distinguished a group of 45 strains that were phylogenetically close. This group is composed of 36 strains corresponding to LAM10/Cameroon (ST 61) strains and nine additional strains related to ST 61 (ST 772 for five strains, ST 57 for two strains, and two orphan strains). The 75 other strains isolated in this region do not present a clear dominant group (Fig. 1).

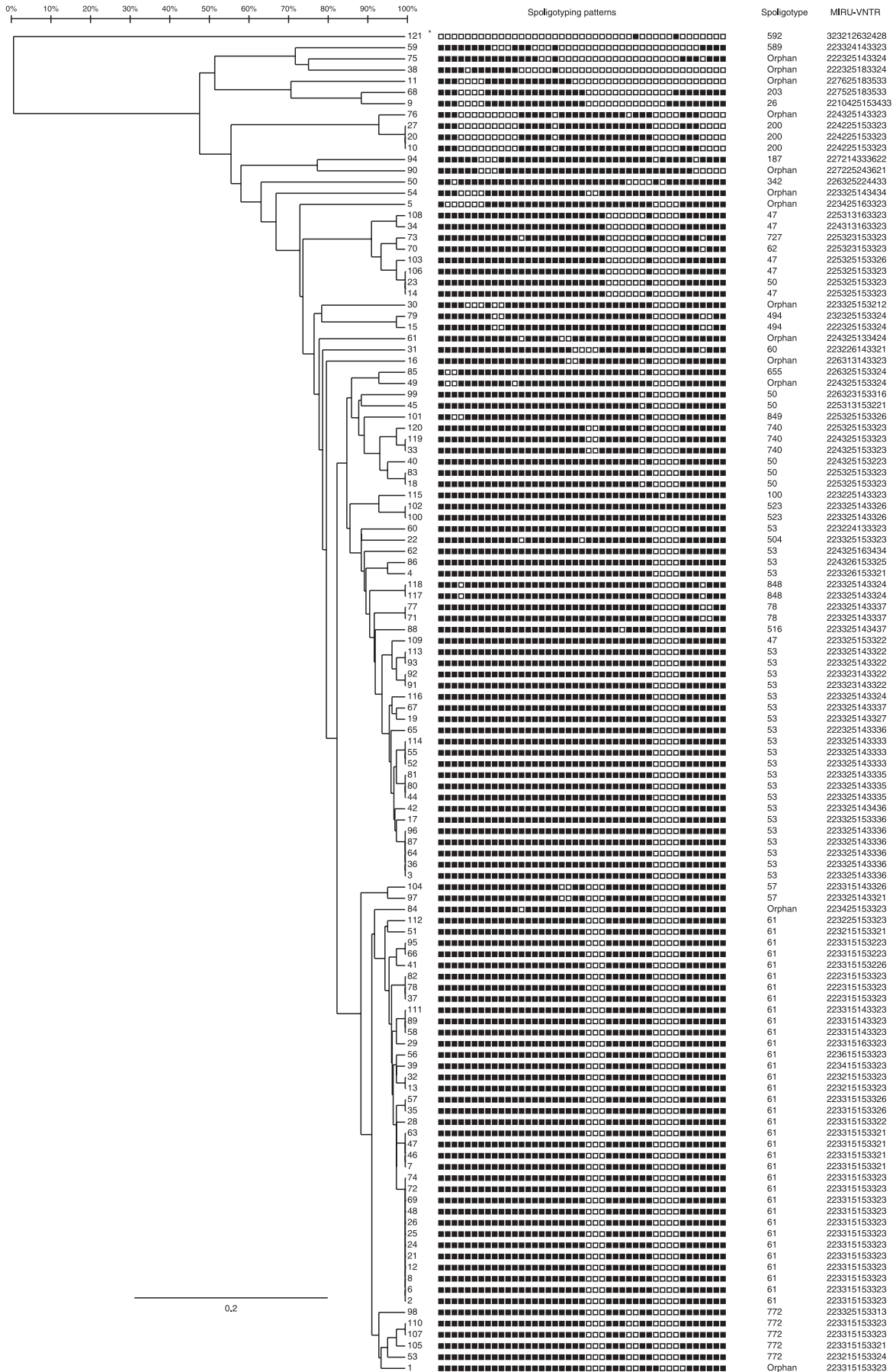
**Factors associated with clustering.** We considered here the most discriminative typing method, i.e., the combined data of MIRU-VNTR and spoligotyping techniques. We studied the distribution of different epidemiological parameters as a function of clustering (64 clustered versus 56 nonclustered strains). In univariate analysis, characteristics such as age (<35 years), male sex, HIV status, resistance to drug, and town of origin (Bobo-Dioulasso or Ouagadougou) showed no association with strain clustering (Table 1).

We compared the distribution of spoligotype families in clustered and nonclustered groups identified by MIRU-VNTR plus spoligotyping. Univariate analysis showed that patients infected by strains belonging to the LAM10 family were significantly associated with clusters (29 LAM10 strains were clustered [80.5%], whereas seven LAM10 strains were nonclustered [19.5%];  $P = 1.7 \times 10^{-7}$ ). The distribution of other spoligotype families (Haarlem, T, numerical, or orphan) did not present a significant association with clustering.

## DISCUSSION

Although Burkina Faso presents a high tuberculosis incidence, no molecular studies have been conducted to date. To explore the molecular epidemiology in this country, we chose to work with two effective and rapid techniques: MIRU-VNTR plus spoligotyping. As in studies of isolate populations from other countries, we found that the combination of the two techniques provided a higher discriminatory power than did the two techniques taken individually (6, 16, 27). On this basis, spoligotyping was primarily utilized here to identify *M. tuberculosis* families, while the combined MIRU-VNTR and spoligotyping data were used to assess their consistency and the clonal diversity within these families.

All TB cases reported in this study were caused by *M. tuberculosis*. The molecular investigation of the Burkina Faso strains by spoligotyping did not show the specific signature of *M. africanum* and *Mycobacterium bovis* (20). The absence of *M. africanum* is somewhat unexpected since the studies by Simonet et al. in 1989 and Ledru et al. in 1996 showed that *M.*



*africanum* was responsible for 18% of human tuberculosis cases in Burkina Faso (17, 25). Nevertheless, the same phenomenon has been observed in Cameroon (20). Indeed, in 1971 Huet et al. showed that 56% of human tuberculosis cases were caused by *M. africanum* (13), while in 2003 Niobe-Eyangoh et al. reported a rate of 9% (20). These data may reflect a decrease in *M. africanum* prevalence in these countries. Nevertheless, the factors that contribute to the reduction of this species have not been unraveled.

Concerning *M. bovis*, despite the high prevalence of bovine TB in Burkina Faso among cattle (31), the studies by Rey et al., Vekemans et al., and Ledru et al. showed a low rate of *M. bovis* in humans (17, 22, 31). These results are in agreement with the absence of *M. bovis* in our study. A recent study conducted in Cameroon showed only one case of pulmonary TB caused by *M. bovis* out of 455 cases (20). The low rate of human pulmonary TB caused by *M. bovis* in our study could be explained by different factors: (i) a high number of the *M. bovis* infections are responsible for extrapulmonary TB cases and in our study the TB cases were essentially pulmonary cases or (ii) pulmonary TB due to *M. bovis* may be more frequent in rural areas (31) and the majority of the patients in our study come from urban areas (Bobo-Dioulasso and Ouagadougou).

The comparison of spoligotyping from Burkina Faso with the International Spoligotyping Database showed that the three major clusters belong to major clades of *M. tuberculosis* (Haarlem, 9%; LAM, 30%; and T, 20%). The Haarlem family (STs 50 and 47) and T family (ST 53) are highly prevalent in West Africa (20) but also worldwide. The LAM family spoligotypes described in our study are specifically identified as LAM10 types (ST 61). This genotype has a limited geographical distribution in countries on the West African coast (Cameroon, Benin, Senegal, and Ivory Coast). The statistical analysis demonstrated that this type is significantly associated with clusters, suggesting its preponderant role in recent transmission in Burkina Faso. Similar results were reported for Cameroon by Niobe-Eyangoh et al. (20). They identified a group of strains principally made up of LAM10 types, which they designated as the Cameroon family. This Cameroon family has also been associated with recent transmission of TB in Burkina Faso (20). Our data in combination with these last data may suggest the recent expansion of this family in West Africa. Nevertheless, the factors that contribute to the success of this family have not yet been unraveled.

Among the spoligotypes isolated in Burkina Faso, STs 848 and 849 were also described in Cameroon and corresponded to three of our strains. Of the orphan spoligotypes absent in SpolDB4, some of them present spoligotype patterns close to the major family. Others show original spoligotype patterns not related to major spoligotypes.

A large number of studies have identified multiple factors associated with TB associated with recent infection, including young age (1, 21, 26), HIV infection status (1, 26), and drug

resistance (1, 9). In our univariate analysis, no associations between clustering and epidemiological data (age, sex, HIV status, and geographical location) were identified (Table 1). The published HIV data seem to demonstrate that HIV status would be statistically associated with clustering in industrialized countries (1, 26, 29), whereas in developing countries and especially in Africa, despite the high rate of HIV infection, several studies reported no association of HIV status with clustering (11, 12, 18, 33).

The high number of small clusters could reflect the importance of recent transmission in Burkina Faso but suggests that these strains cannot propagate in the population. Indeed, the relatively efficient control of the disease in this area limits the propagation of these genotypes. Besides these numerous small clusters, we observed high global heterogeneity also due to the high number of unique genotypes. These genotypes may correspond to TB reactivation cases or migration phenomena. The high mean genetic diversity observed in Burkina Faso could reflect the balance between a high level of clustering (recent transmission) and heterogeneity corresponding to endogenous reactivation and migrations. Only a study integrating the majority of tuberculosis cases will provide an adequate estimation of the balance between recent transmission and reactivation.

In the present study, drug resistance to one or more anti-TB drugs was found in 5% of the tested strains. Since all the isolates were taken from never-treated patients, the drug resistance found in the present study can be considered primary drug resistance. The data already published concerning primary resistance show a clear decrease between 1986, 1994, and 2001 (17, 25): isoniazid, 19.5% in 1986, 7.6% in 1994, and 4.9% in 2001; streptomycin, 23.9% in 1986, 12.4% in 1994, and 0% in 2001; rifampin, 2.5% in 1994 and 1.6% in 2001. The level of primary resistance in our study also appears to be lower than levels observed in other African countries (13.4% in Ivory Coast in 1995 to 1996, 31.8% in Cameroon in 1995, and 9.6% in Malawi in 1986 to 1998) (3, 7, 32). Despite the small number of tested samples (61 strains), the lack of streptomycin-resistant strains and the low level of isoniazid resistance are surprising. Indeed, many studies showed that resistance to streptomycin and isoniazid was most frequent in sub-Saharan Africa (2, 7, 10). The high rate of resistance to these drugs is related to the intensive use of low-cost or free drugs (2, 7). Furthermore, in some countries such as Cameroon, streptomycin is widely used to treat other infections and this can explain the rate of streptomycin-resistant strains (7). From our data, the low rate of primary drug resistance could be explained either by optimal medical management of TB or a lack of treatment. But all these assumptions must be assessed from a more significant number of *M. tuberculosis* strains.

In conclusion, our investigation of DNA polymorphism of *M. tuberculosis* complex strains from humans in Burkina Faso has shown the *M. tuberculosis* species to be the sole agent of TB

FIG. 1. UPGMA tree based on combined data, spoligotypes, and MIRU-VNTR of the 120 samples under study (an *M. canettii* stock is used as the outgroup [asterisk]). The relationships between patterns were assessed by the unweighted-pair group method using average linkages. The spoligotypes listed correspond to the designations in the SpolDB4 database (5). Orphan patterns match none of the spoligotypes described in the SpolDB4 database.

cases. The data obtained in this study show the predominance of the LAM10 family, called the Cameroon family by Niobe-Eyangoh et al. (20), mainly implicated in cases of recent TB transmission. The same phenomenon has been observed in Cameroon, suggesting an emergence of this *M. tuberculosis* family in the countries of West Africa. Further studies are needed to understand and to follow the expansion of this predominant family in West Africa. Our findings of a relatively high proportion of clusters suggest that recent transmission is an important cause of the rising incidence of TB in Burkina Faso. Besides, the important genetic polymorphism reflects a situation of endemicity in Burkina Faso where the control of the disease would restrain the expansion of TB in particular in the main town. No risk factor including HIV status, male sex, age (<35 years), drug resistance, or town of origin was associated with clustering in our sample. Our finding of a high proportion of TB cases from HIV-positive individuals (35.8%) underlines the importance of medical management of HIV. Furthermore, our data suggest a low level of primary drug resistance, which may reflect the successful Directly Observed Therapy Shortcourse program.

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