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Tuberculosis transmission in a high incidence area: A retrospective molecular epidemiological study of *Mycobacterium tuberculosis* in Casablanca, Morocco

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Abstract

Like in most developing countries, tuberculosis represents a major public health problem in Morocco. This paper describes the first study combining molecular and conventional epidemiology of tuberculosis in Casablanca, the economic capital of this country. Molecular fingerprinting of the genomic DNA recovered from cultures of sputum of 150 patients was performed by MIRU-VNTR. This molecular marker revealed that 53.1% of the total cases were clustered. These cases were classified into 23 clusters ranging in size from 2 to 13 patients, suggesting a rate of 37% of recent transmission in the sample under study. In a multivariate analysis, there were no independent predictors of clustering. However, the clinical form was associated with drug resistance (odds ratio = 9.9; *P* value = 0.0006). The phylogenetic analysis showed that the heterogeneity found in this population includes also the members from a same patient family, and that the 2 majoritary families distributed in Casablanca were the Latin–American–Mediterranean (LAM) and Haarlem families. All the results of this work allow to understand better the tuberculosis transmission in Casablanca, and suggest that different clones of *M. tuberculosis* seem to circulate in this city, and that the reactivation of latent infections would be mainly responsible for the endemic situation of this disease. These findings indicate also that the transmission of TB in Morocco is not optimally controlled, and that efforts for control strategies should be sustained in all developing countries where the incidence of TB is high and still raising.

Keywords: Tuberculosis; Molecular epidemiology; Morocco; MIRU-VNTR

1. Introduction

Tuberculosis (TB) still represents an important global public health threat; it is one of the world's leading causes of death, killing more than 2 million people annually (Raviglione et al., 1995; Dye et al., 1999). The global resurgence of TB had been mainly related to the increase of HIV/AIDS prevalence, emerging anti-tuberculosis drug resistance, and also to the inadequate investments in public health systems predominantly in developing countries (Grange and Zumla, 2002; Frieden et al., 2003; Kodmon et al., 2006; Owusu-Dabo et al., 2006).

In Casablanca, the economic capital of Morocco, the incidence of TB is very high with nearly 160 new cases per 100,000 inhabitants each year. This city includes almost the fifth of the cases recorded in this country (National Anti-Tuberculosis Fight Program, Department of Health, Rabat, Morocco). Even though Morocco has adopted the Directly Observed Treatment Short-course (DOTS) program as the main strategy for TB control since 1991, the incidence is still increasing (Ottmani et al., 1998; Laraqui et al., 2001). Population genetic studies remain limited in Morocco (El Baghdadi et al., 1997a,b; Diraa et al., 2005); up to now only one extensive study was conducted in Casablanca and showed an unexpected genetic diversity with a basically clonal structure for this population in such a high incidence community (Tazi et al., 2004). Moreover, the pattern of TB transmission (reactivation versus recent infection) and the risk factors

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associated with transmission dynamics of this disease are still to be determined.

In recent years, DNA fingerprinting of *Mycobacterium tuberculosis* has revolutionized our understanding of TB transmission (McNabb et al., 2002; Murray and Nardell, 2002; Barnes and Cave, 2003). Epidemiological studies of TB have been greatly assisted by the advent of Restriction Fragment Length Polymorphism (RFLP) typing using the insertion sequence IS6110 (Cave et al., 1991; Diel et al., 2004; Kempf et al., 2005; Kodmon et al., 2006). This technique is considered as the gold standard typing method, but it presents some disadvantages (Kremer et al., 1999; Cowan et al., 2002). Another fingerprinting technique based on Variable Number of Tandem Repeats (VNTRs) in eukaryotic-like minisatellites has been recently described in the *M. tuberculosis* genome (Supply et al., 2000; Mazars et al., 2001). These elements have been named Mycobacterial Interspersed Repetitive Units (MIRUs), and showed their potential for strain typing and for molecular epidemiology of tuberculosis in different studies (Mazars et al., 2001; Cowan et al., 2002; Sun et al., 2004; van Deutekom et al., 2005; Loiez et al., 2006).

In the present work, we used MIRU-VNTR typing to understand the transmission of TB in Casablanca, to compare the Moroccan population with different *M. tuberculosis* families, and to determine risk factors associated with recent transmission. This work represents the first study combining molecular and conventional epidemiologic approaches in a city of high prevalence in Morocco.

2. Materials and methods

2.1. Mycobacterial isolates and clinical data

The study includes 155 isolates of *M. tuberculosis* mostly collected in 1997 and 1998, with five isolates representing follow-up of five different patients. These isolates are obtained from sputum of 150 tuberculous patients (see Table S1 in Supplementary data). They come from six different districts of Casablanca: HMAS ''Hay Mohammadi Aïn Sebaa'', BMSO ''Ben Msik Sidi Othmane'', CA ''Casablanca Anfa'', FDS ''Fida Derb Sultan'', HHAC ''Hay Hassani Aïn Chock'', and MOH ''Mohammedia'', except one isolate (isolate 97; see Table S1 in Supplementary data) which comes from Benslimane, a city distant of about 50 km from Casablanca. Three additional isolates were collected in Casablanca without information about the district of origin. The total sampling under study was obtained in the year 2000, and most of the isolates were collected from patients living in the Casablanca district HMAS, which represents one of the most prevalent districts in this city for TB incidence (National Anti-Tuberculosis Fight Program, Department of Health, Rabat, Morocco). Like most developing countries, all the reported cases of TB are not systematically cultured, which limits therefore our collection. However, by considering different isolates covering different districts of Casablanca, the genetic analysis of such sampling provides sufficient data for exploring the transmission dynamics of TB in this city.

For five patients, two isolates per individual were taken at different times (3–9 months during treatment) corresponding to the follow-up of the disease (isolates number: 5 and 5′, 41 and 41′, 56 and 56′, 119 and 119′, 126 and 126′; see Table S1 in Supplementary data). Seven families whose members were infected by *M. tuberculosis*, were also included in this study (family 1: isolates 20, 21, 51; family 2: isolates 25, 55; family 3: isolates 26, 56, 153; family 4: isolates 34, 37, 58; family 5: isolates 52, 53; family 6: isolates 118, 119; and family 7: isolates 131, 132, 133) (see Table S1 in Supplementary data).

Different forms of TB were also represented in our sample, including primary resistance, multi-drug resistance, relapses, new cases, and chronic TB. For each isolate, epidemiological data were collected in the different health centers specialized on TB in each district (CDST: Centre de Diagnostic Spécialisé de la Tuberculose).

All the isolates included in this study were recovered by culture on Löwenstein–Jensen medium. The identification of these mycobacteria as *M. tuberculosis* was performed by conventional biochemical methods. For each isolate, the resistance profile to Isoniazid ''H'', Rifampin ''R'', Streptomycin ''S'', and Ethambutol ''E'' was tested, according to the conventional proportion method (Canetti et al., 1963). This method is based on the estimation of the proportion of resistant strains in a given isolate. The concentrations of the anti-tuberculosis drugs used were as follows: Isoniazid, 0.2 mg/l; Rifampin, 40.0 mg/l; Ethambutol, 2.0 mg/l; and Streptomycin, 4.0 mg/l. An isolate is considered sensitive if the proportion of resistant strains is less than 1% for these anti-tuberculosis drugs.

Also in order to compare the Moroccan population with *M. tuberculosis* families (Africa, Haarlem, W-Beijing, Latin–American–Mediterranean, and East-African-Indian) and to characterize the prevalent family in Casablanca, the MIRU data from 69 isolates of *M. tuberculosis* were included in our analysis (Kremer et al., 1999; Supply et al., 2001).

2.2. Isolation of chromosomal DNA and MIRU-VNTR PCR

Extraction of DNA from all Moroccan *M. tuberculosis* isolates was performed by a standardized protocol described previously with slight modifications (van Soolingen et al., 1994). The quantity of each DNA was evaluated by measuring the optic density at 260 nm, and its quality was checked on agarose gel (0.8%).

The MIRU technique is based on PCR amplification of Variable Number of Tandem Repeats (VNTRs) of genetic elements named Mycobacterial Interspersed Repetitive Units (MIRUs), in 12 loci referred to as MIRU 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40 (Supply et al., 2000). The amplifications were performed by using the *HotStarTaq* DNA polymerase kit (Qiagen, Hilden, Germany) and oligonucleotides corresponding to the flanking regions of the 12 MIRU-VNTR loci, as described previously with slight modifications (Supply et al., 2000, 2001). A 10 ng of DNA (10 ng/μl) was amplified in a total volume of 50 μl containing *HotStarTaq* DNA polymerase buffer 1×, 0.2 mM of each dNTP, primer 0.4 μM, Q solution 1×, *HotStarTaq* DNA polymerase 20 mUI/μl, and $MgCl_2$

1–3.5 mM. The program of amplification started with a step of 15 min at 95 °C, followed by 40 cycles (1 min at 94 °C, 1 min at 59 °C, 1 min 30 s at 72 °C). An extension step (7 min at 72 °C) ended this program. The amplicons were analyzed by electrophoresis by using 2% NuSieve agarose. The estimation of the size of the PCR fragments was established by comparison with 20- and 100-bp superladders-low (Eurogentec, Seraing, Belgium). The conversion of the size of the amplicons into the number of MIRUs per locus was done by using the conventions described previously (Supply et al., 2000; Mazars et al., 2001). The resulting numerical genotype codes correspond to the numbers of VNTR in each of the 12 loci (Mazars et al., 2001).

2.3. Phylogenetic analyses

Phylogenetic relationships among the isolates were inferred from MIRU data by using Neighbor–Joining analysis (Saitou and Nei, 1987) with bootstrapping (Efron, 1979) in order to test the robustness of the nodes. These analyses were computed using the Genetics Toolbox and Treedyn softwares (Chevenet et al., 2006) designed in our laboratory, and the PHYLIP software (Felsenstein, 1993, 3.5c ed. Seattle, WA. Department of Genetics, University of Washington). We used *M. canettii* as outgroup for these analyses (Kremer et al., 1999).

2.4. Statistical analyses

All patients included in this study were classified in two groups characterized by clustered and non-clustered *M. tuberculosis* isolates. A cluster was defined as two or more patients' strains with identical genetic patterns, and patients' strains with unmatched genetic profiles were considered non-clustered. Clusters were assumed to have arisen from recent transmission, and the clustering rate was used to determine the amount of recent transmission in this population (Small et al., 1994). The patients' strains with the same genetic pattern represent an epidemiologically linked cluster. Therefore, the minimum estimate of the proportion of TB cases related to recent transmission was calculated as (number of clustered patients – number of clusters)/total number of patients. Variables that could have been associated with either clustering or drug resistance of the pathogen (gender, year of birth, year of diagnosis, and clinical form) were first tested in a bivariate analysis, and were then included in multivariate logistic regression models in order to test for independent risk factors for recent transmission. The data were analyzed using EPI Info™ (Dean et al., 2002), and the models were built with different sets of variables using a stepwise elimination method based on the likelihood-ratio statistic (Hosmer and Lemeshow, 1989). *P* values below 0.05 were considered statistically significant.

3. Results

3.1. Study population

One hundred and fifty patients with pulmonary TB were analyzed in this study by MIRU-VNTR typing (see Table S2 in Supplementary data). All these cases were culture positive. Among this population, three patients had no epidemiological record, and they were therefore excluded for the study population in addition to the follow-up. The majority of the isolates were collected from patients living in the district HMAS in Casablanca (66%), of which 44 (45.4%) were diagnosed in 1997, and 30 (30.9%) in 1998. The other patients came from the following districts: BMSO (15%), CA (10.9%), FDS (2%), HHAC (4.1%), MOH (1.4%), and one isolate from Benslimane (0.7%). The male-to-female ratio in the study population was 2.2:1. The patients were from 6 to 77 years old (mean age = 39 years, median age = 33 years), most of whom (77%) were between 18 and 40 years old.

Ninety-four isolates (65.3%) were susceptible to all four drugs tested (Rifampin "R", Isoniazid "H", Streptomycin "S", and Ethambutol "E"). Twenty-two percent were resistant to one drug, with 8% showing primary resistance to Isoniazid or Streptomycin, and 4% and 2% showing secondary resistance to Rifampin and Streptomycin, respectively. Twenty-two percent of the isolates were resistant to two drugs, with 14% and 2% showing primary resistance to Isoniazid/Streptomycin and to Rifampin/Streptomycin, respectively, and 6% and 4% showing secondary resistance to Isoniazid/Streptomycin and Rifampin/Streptomycin, respectively. The multi-drug resistant (MDR) strains represent 56% of the total resistant isolates. In the population under study, 79.6% of the isolates were reported as new cases whereas 17.7% and 2.7% of the isolates represented chronic cases and relapses, respectively.

3.2. MIRU-VNTR analysis

The complete collection was considered for this analysis, and the molecular marker showed a high polymorphism in this population (Fig. 1). Seventy-eight cases (53.1%) shared an identical MIRU profile with one or more other patients (clustered cases) and were classified into 23 clusters ranging in size from 2 to 13 people, of which 13 (56.5%) included just two patients (Fig. 2). Our data suggested that recent transmission accounted for 37% of tuberculosis cases.

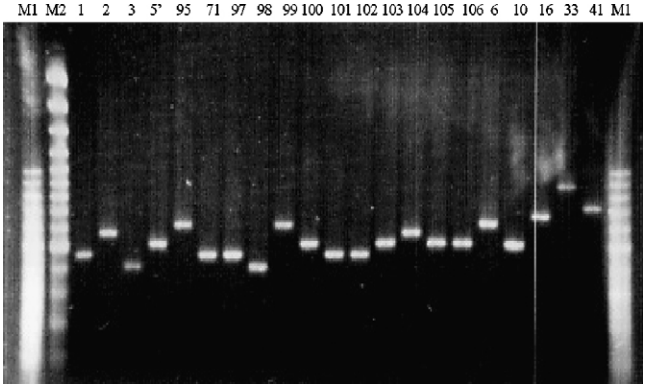

Fig. 1. Electrophoretic pattern obtained by PCR-MIRU with the locus 40 for some Moroccan isolates. Lane numbers correspond to codes of isolates (see Table S1 in Supplementary data). M1 and M2 correspond, respectively, to 20–100 pb and 100 pb superladders-low.

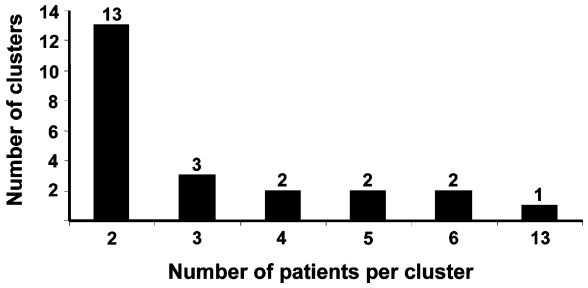

Fig. 2. Number of clusters according to cluster size. Clusters were defined by MIRU-VNTR typing.

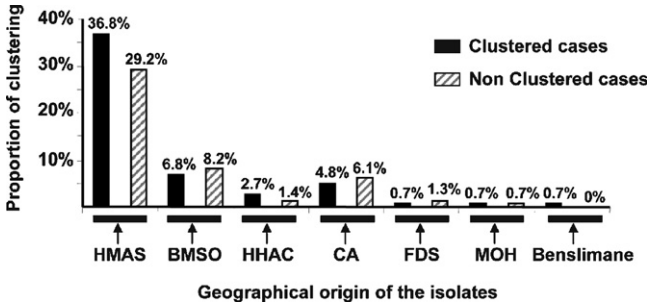

Fig. 3. Distribution of clusters in the data collection according to the geographical isolation.

Among the 78 patients grouped in clusters, 54 cases (36.8%) came from the district HMAS. The district BMSO had only 10 clustered cases (6.8%), whereas the districts CA and HHAC comprised 7 (4.8%) and 4 (2.7%) clustered cases, respectively. The isolates from the other districts FDS and MOH and the city of Benslimane showed only one clustered case (0.7%) in the analysis (Fig. 3).

The five pairs of isolates collected at different times from a same patient displayed exactly the same MIRU profiles, consistent with results indicating the stability of MIRU-VNTR profiles in serial isolates from French and South African patients (Supply et al., 2001; Savine et al., 2002). In order to avoid a bias of considering twice the same isolate in our study, for the phylogenetic and statistical analyses, only one isolate by patient (isolates 5′, 41, 56, 119, and 126) was used.

Two situations were found among seven families with different patient members living in the same household. In only three cases, isolates from different members of the same family displayed identical genotypes. The isolates from the members of the four other families showed different profiles of MIRU-VNTR. For example, the isolates of family 4 (isolates 34, 37, 58) have shown different profiles with 5 loci MIRU (MIRU 2, 10, 20, 23, and 40) (data not shown).

3.3. Phylogenetic analyses

From the phylogenetic trees constructed, we could distinguish some isolates that shared similar genetic patterns and were therefore grouped together. However, none of these groups was associated with either geographical origin, or biological properties or clinical characteristics of the patients under study. Indeed, the phylogenetic analysis performed for example on the population isolated in HMAS district showed that the members of the same family were not always grouped together and did not share the same MIRU-VNTR profiles (Fig. 4). Moreover, the MDR strains were mixed with the other strains (data not shown).

Our phylogenetic analysis included also a comparison of the Moroccan population with some *M. tuberculosis* families. In this case, we found that the 2 majoritary families mixed with the Moroccan population were the families Latin–American–Mediterranean (LAM) and Haarlem (Fig. 5).

3.4. Characterization of risk factors associated with recent transmission

In the bivariate analysis, none of the variables (gender, year of birth, year of diagnosis, clinical form, and drug resistance) was significantly associated with clustering (data not shown). However, the drug resistance showed a significant association with the following variables: year of birth, year of diagnosis, and clinical form (P value ≤ 0.001), except for gender (P value = 0.699) and clustering (P value = 0.626).

In the logistical regression, the independent variable found to represent the highest predictive risk in drug resistance was the clinical form (new case versus chronic TB versus relapse). In fact, this variable (clinical form) showed a strong association controlled for year of birth, year of diagnosis, and clustering, with a 10-fold increase in risk (odds ratio = 9.9; P value = 0.0006) (Table 1). However, no risk factor associated with recent transmission for the patients included in a cluster was identified (data not shown).

4. Discussion

Knowledge on *M. tuberculosis* population structure and tuberculosis transmission still remains poor in many regions with a high incidence of tuberculosis. This work represents a retrospective study in Morocco; it is the first study that focuses on the identification of risk factors associated with recent transmission of tuberculosis in this country, and it complements the previous population genetics study conducted in the same area (Tazi et al., 2004).

Among the 155 Moroccan isolates, 154 have been selected randomly in different districts of Casablanca, 101 of which were isolated from a district (HMAS) with a high incidence of pulmonary tuberculosis of about 450 new cases per year (National Anti-tuberculosis Fight Program, Department of Health, Rabat, Morocco). Therefore, this sample, although not exhaustive, is a good retrospective basis for molecular epidemiology of tuberculosis in Morocco, and particularly in Casablanca. In fact, the culturing of confirmed TB cases is not systematically performed in this country because of the cost of these analyses, and the population under study represents the complete collection that was available during the establishment of this project. This situation is very common in developing countries, and it limits considerably the number of the sampling. However, this collection is distributed randomly in

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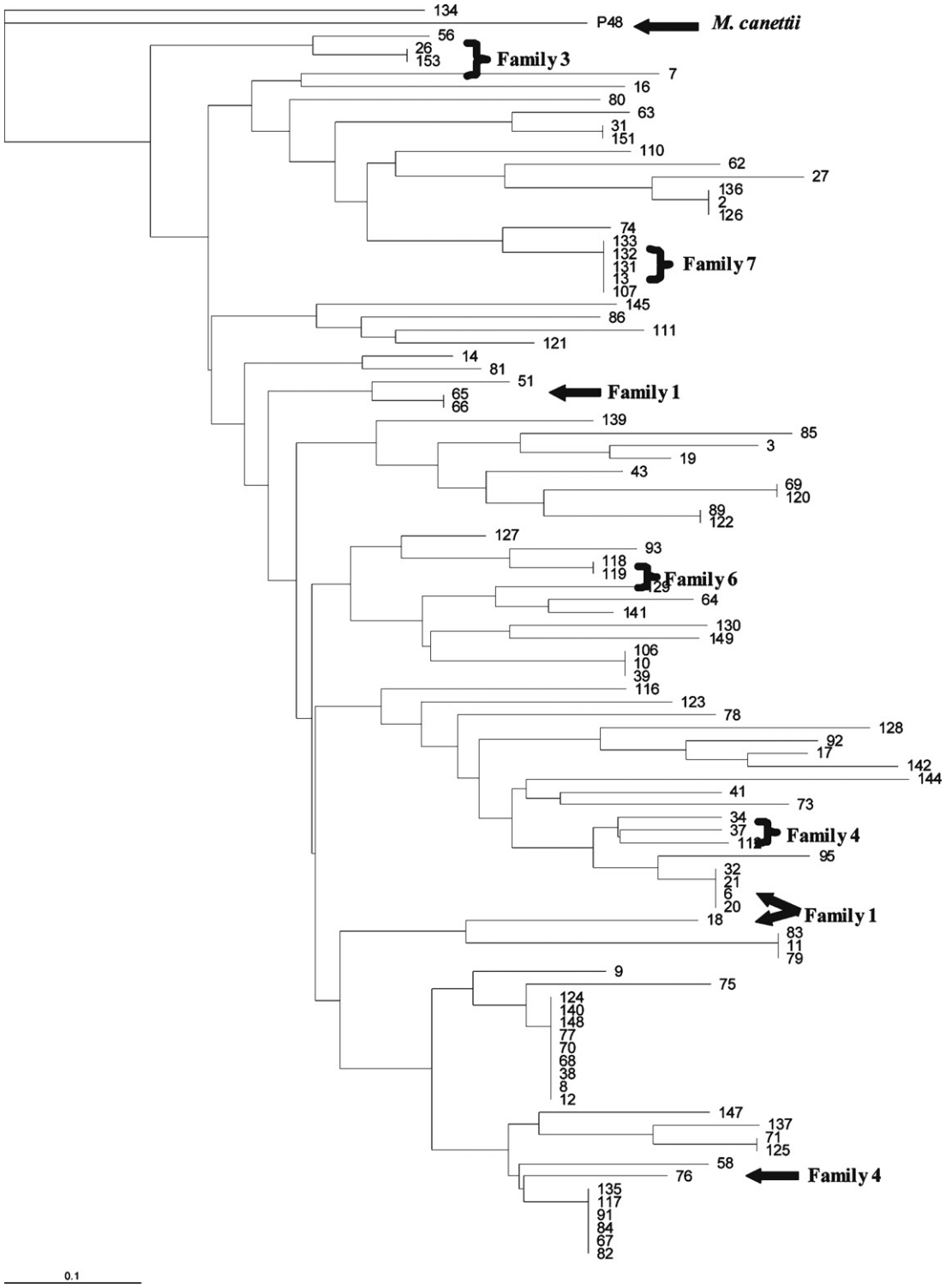

Fig. 4. Neighbor–Joining tree performed on the population isolated in HMAS district. *M. canettii* stock is used as outgroup. The seven Moroccan families included in this study are also included in this tree.

the city of Casablanca, and will therefore reflect the transmission scenario of TB in this setting.

Among the patients under study, clustered isolates represented half of the total isolates (53.1%) with the twelve MIRU-VNTR loci used. This result indicates a rate of recent transmission of 37% in this city. Moreover, if we consider the HMAS district individually, the clustering rate was 55.7% for all the patients living in this district. These patients were classified into 21 clusters, and the rate of recent transmission in this district was estimated to be 34%. These rates are lower than estimates of recent transmission and rates of clustering reported in other studies performed in high incidence TB areas (Easterbrook et al., 2004; Verver et al., 2004b; Brudey et al., 2006). In fact, the proportions of new cases due to recent

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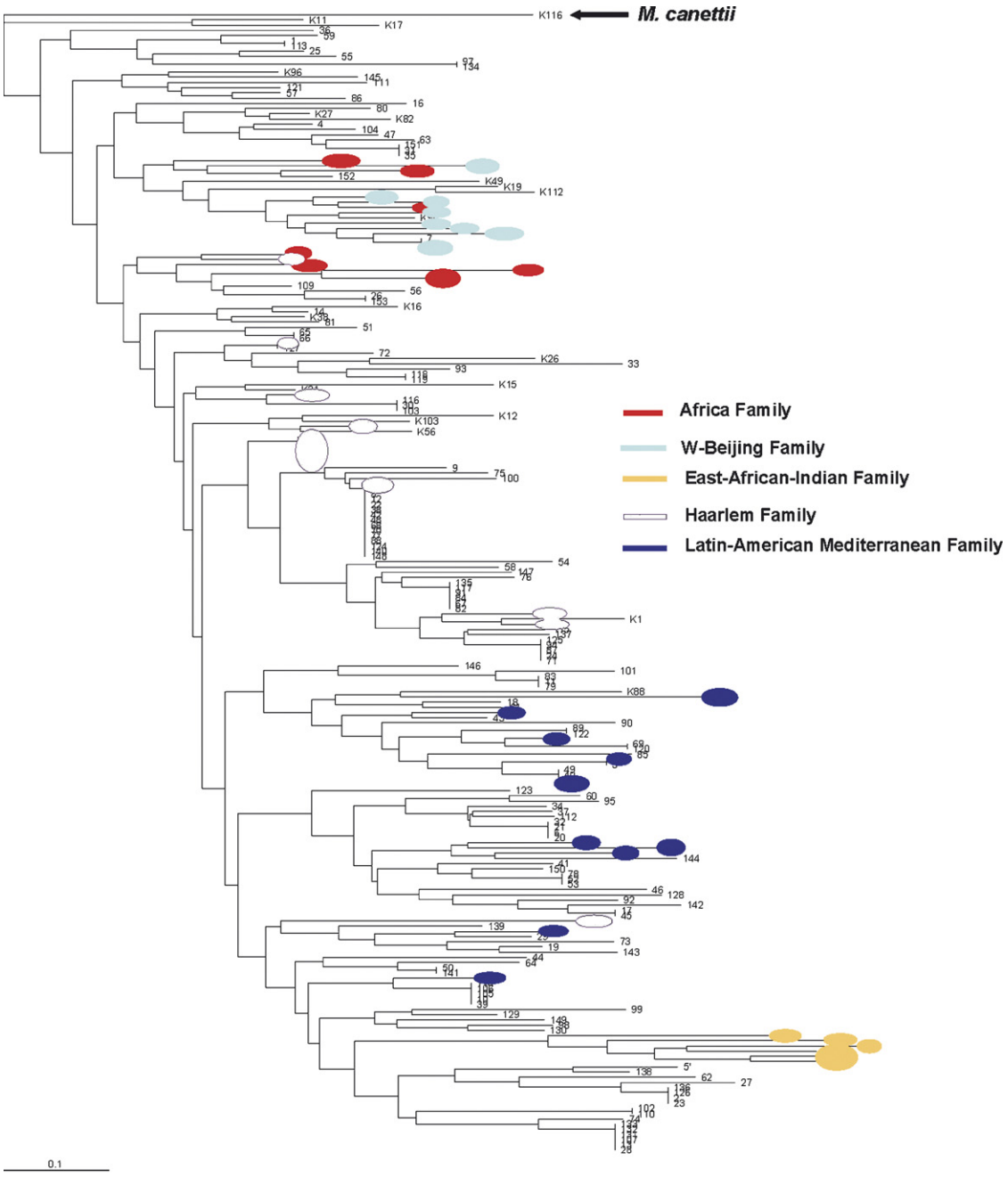

Fig. 5. Neighbor–Joining tree based on MIRU-VNTR data and presenting the relationships between the Moroccan population and some *M. tuberculosis* families. *M. canettii* stock is used as outgroup.

transmission have been found to be close to the ones found in areas where the rates of TB are low (Small et al., 1994; Bauer et al., 1998; Bishai et al., 1998; Gutierrez et al., 1998; Kempf et al., 2005). Moreover, according to the data published by Supply et al. (2006), it is possible that the rate of clustering and recent transmission estimate in this study have been over-estimated due to the number of MIRU-VNTR loci used (12 loci instead of 15 or 24 loci). Indeed, these authors have recently demonstrated that the number of MIRU-VNTR loci can strongly influence the calculation of recent transmission rate. Thus, it strongly suggests that the rate of recent transmission calculated here could be weaker. This overestimation reinforces the report that the rate of recent transmission in Casablanca is still lower than the ones estimated in the countries with high incidence of TB. Taken this comparison into account, the transmission pattern may also differ in our setting, and the clustering rates should be interpreted carefully because areas

Table 1
Risk factors for recent transmission associated with drug resistance

Risk factor	Odds ratio	95% CI	*P* value
Year of birth	0.9795	0.949–1.011	0.2003
Year of diagnosis	0.9291	0.786–1.099	0.3904
Clinical form	9.8768	2.682–36.373	0.0006
Clustering	1.3305	0.589–3.001	0.4914

CI: Confidence interval.

that showed higher recent transmission and clustering rates are usually either known to have a very high prevalence of HIV and/or are subject to a TB outbreak responsible for an epidemic situation but not endemic case of TB in these countries. However, this finding cannot be the result of sampling bias because we will not expect to have a higher recent transmission and clustering rates by recovering the total cases of TB registered in Casablanca. Also, the significance of clustering in population-based studies is controversial. Several studies have shown that the isolates belonging to the same cluster may occur by chance through coincidental reactivation of latent infections during the observation period (Bennett et al., 2002; Diel et al., 2002). This could be the case of TB transmission in Casablanca, since no risk factor was associated with recent transmission for the patients belonging to a cluster. However, the logistic regression model showed that the clinical form represented a considerable risk with drug resistance (odds ratio = 9.9; P value = 0.0006).

In Morocco as well as in other Southern Mediterranean or Middle East countries, the classical risk factors, AIDS disease and alcohol and drug abuse, are not significantly implicated in the increase of the disease. Several studies showed that the high prevalence of HIV infection was responsible for high rates of clustering in different countries (Gilks et al., 1997; Wilkinson et al., 1997; Easterbrook et al., 2004). However, the incidence of HIV is still very low in Morocco; the cumulative number of AIDS cases is around 15,000 (Elharti et al., 2002), and this situation could also explain the relatively low clustering found in the population from Casablanca. In this city, tuberculosis mainly affects much wider defective socio-economic groups.

Moreover, our results indicate that members of a same patient family were often contaminated by different strains. The latter result is consistent with observations in other high incidence areas indicating that, within families with several diseased members, transmission of tuberculosis may originate from contacts outside the household (Verver et al., 2004b). This factor additionally complicates identification of sources of infections by classical epidemiological investigation.

Despite the clonal evolution inferred for this bacteria and the notable genetic diversity found in the Moroccan *M. tuberculosis* population (Supply et al., 2003; Tazi et al., 2004), the phylogenetic analyses revealed no grouping for the isolates with a drug resistance profile or clinical characteristics of the disease (chronic tuberculosis, new cases, and relapses) on one hand and the genetic marker used on the other hand. This suggests first that immunological and physiological status of patients plays without doubt a role in the expression of the disease, and second that the genetic determinants of mycobacteria linked to the drug resistance would evolve independently of MIRU-VNTR markers. Also, these analyses showed that by comparing the Moroccan population with some *M. tuberculosis* families, there are 2 majoritary families dispersed in Casablanca accompanied by a large genetic heterogeneity, and this result confirms the biodiversity present in this city.

In contrast with other regions with high incidence (Calusni et al., 2003; Verver et al., 2004a), the phylogenetic analysis showed also no major multilocus genotypes in this sample, even when the HMAS district of Casablanca or the year of the isolation were considered alone. Taken together with the relatively low degree of clustering described in this study, these observations suggest that reactivation of old latent infections may be the main driving force for the endemic situation of the disease in Casablanca. Nevertheless, recent transmission of tuberculosis in this city is non-negligible since the analysis with the present sample suggests 37% of recent transmission rate.

Given our results, several clones seem to be present in Casablanca. Therefore, it would be especially useful to develop routine molecular epidemiological surveillance with the newly discriminatory subset of MIRU-VNTR loci (Supply et al., 2006), in order to better understand the dynamics of the disease in these environments and to optimize current control programs. In fact, these findings indicate also that even though the DOTS strategy has been implemented in Morocco for several years, the transmission of TB is still not optimally controlled, and that efforts for control strategies should be sustained in all developing countries where the incidence of TB is high and still raising. Moreover, it will be important to conduct further studies on *M. tuberculosis* populations from rural areas and other Moroccan cities. Such studies will allow to determine if the situation of tuberculosis in Casablanca is specific to this city, or if a very high polymorphism is detected everywhere in Morocco.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2007.06.005.

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