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Molecular Characterization and Drug Resistance Testing of *Mycobacterium tuberculosis* Isolates from Chad

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The molecular characterizations of the first 40 *Mycobacterium tuberculosis* isolates from Chad revealed a high proportion of isolates of the Cameroon family (33%), of which one isolate showed a monodrug resistance. In total, 9/33 (27%) isolates were resistant to isoniazid. The implications of these findings are discussed.

In Chad, the annual incidence rate of pulmonary tuberculosis was estimated at 60 to 120 of 100,000 in 1990 (12) but increased to 370 of 100,000 in 2000 (19), making Chad a high-incidence country. The recommended WHO treatment strategy for patients with open and extrapulmonary tuberculosis is directly observed chemotherapy, which has been adopted in most African countries, including Chad, where its coverage was 25% in 2003 (20). An increase in drug resistance due to noncompliance during treatment is feared; however, the lack of baseline data on drug resistance from these countries makes monitoring difficult.

Drug resistance testing, molecular characterization, and fingerprinting of *Mycobacterium tuberculosis* complex members have become common practices in most mycobacterial laboratories. A variety of molecular genetic typing tools for *M. tuberculosis* complex isolates have been developed (18), with the most widely used, IS*6110* restriction fragment length polymorphism typing, as the gold standard. More recently, spoligotyping (8) has shown advantages over IS*6110* typing. It is more cost-effective, easier to perform, and possible to compare results between laboratories.

The outcome of the drug resistance tests and molecular characterization by spoligotyping of the first *M. tuberculosis* isolates from Chad are presented and implications for treatment control discussed.

Between March and July 2001 and February and October 2002, a total of 357 sputum and 282 urine samples were collected from tuberculosis patients at the National Reference Hospital (HGRNT) in the Chadian capital, N′Djaména, and at four rural health centers that were 50 to 200 kilometers away from N′Djaména. All specimens (sputum and urine) were decontaminated (9) and inoculated onto two Löwenstein-Jensen slants, one containing 0.75% glycerol and the other containing 0.6% sodium pyruvate. In addition, liquid Middlebrook 7H9 medium containing oleic acid-albumin-dextrose-catalase and polymyxin-amphotericin B-nalidixic acid-trimethoprim-azlocillin was used. The inoculated media were incubated at 37°C without CO_2 for 8 weeks. Smears were made from the sediment and were stained by the Ziehl-Neelsen method (9). Growth of mycobacteria was confirmed by smear. Acid-fast-bacillus-positive colonies were subcultured on three Löwenstein-Jensen slants and a Middlebrook 7H10 agar plate. Three biochemical tests (nitrate, niacin, and 68°C catalase) (9) were used to identify *M. tuberculosis* complex (MTC) from nontuberculous mycobacteria. The Lebek method was used as an additional phenotypic test to distinguish between MTC members (7), and in addition the standard method for molecular identification of MTC isolates, real-time PCR (10), was performed. Genotyping and identification of *M. tuberculosis* isolates were done by spoligotyping (8), and obtained spoligotypes were compared to the international database (SpolDB3.0) (6).

With real-time PCR, 40 MTC-positive isolates were identified, and subsequent spoligotyping resulted in identification of 40 *M. tuberculosis* isolates (Fig. 1). Considering the geographic location of Chad in central Africa, it was interesting to note that spoligotyping revealed no *M. africanum* type I or type II isolates. *M. africanum* type I (lack of spacers 37 to 39) is very frequent in western Africa and is found in neighboring Cameroon. However, it was suggested that there has been a decreasing trend in *M. africanum* type I transmission over the last three decades (16). *M. africanum* type II (lack of spacer 40) is isolated predominantly in Uganda (15), and although we obtained six spoligotypes lacking spacer 40, all of ours were aerophilic on Lebek media, which is not the case for *M. africanum*. Indeed, recent genomic analysis could not differentiate *M. africanum* subtype II from modern *M. tuberculosis*, casting doubt on whether subtype II should be considered separately from *M. tuberculosis* (14). In total, spoligotyping identified 13 strains with the Cameroon family spoligotype (lacking spacers 23 to 25). This family was first described to be a strain family endemic in Cameroon (16) and Nigeria (2), and its presence in Chad is not surprising, since everyday transborder movements of people are frequent.

Drug susceptibility testing was performed with a BACTEC MGIT 960 instrument (BD Biosciences, Sparks, Md.) for isoniazid (INH), 0.1 μg/ml; rifampin (RIF), 1 μg/ml; ethambutol, 5 μg/ml; and pyrazinamide, 100 μg/ml. Streptomycin, 2 μg/ml and 10 μg/ml, was tested by the agar proportion method (9). In

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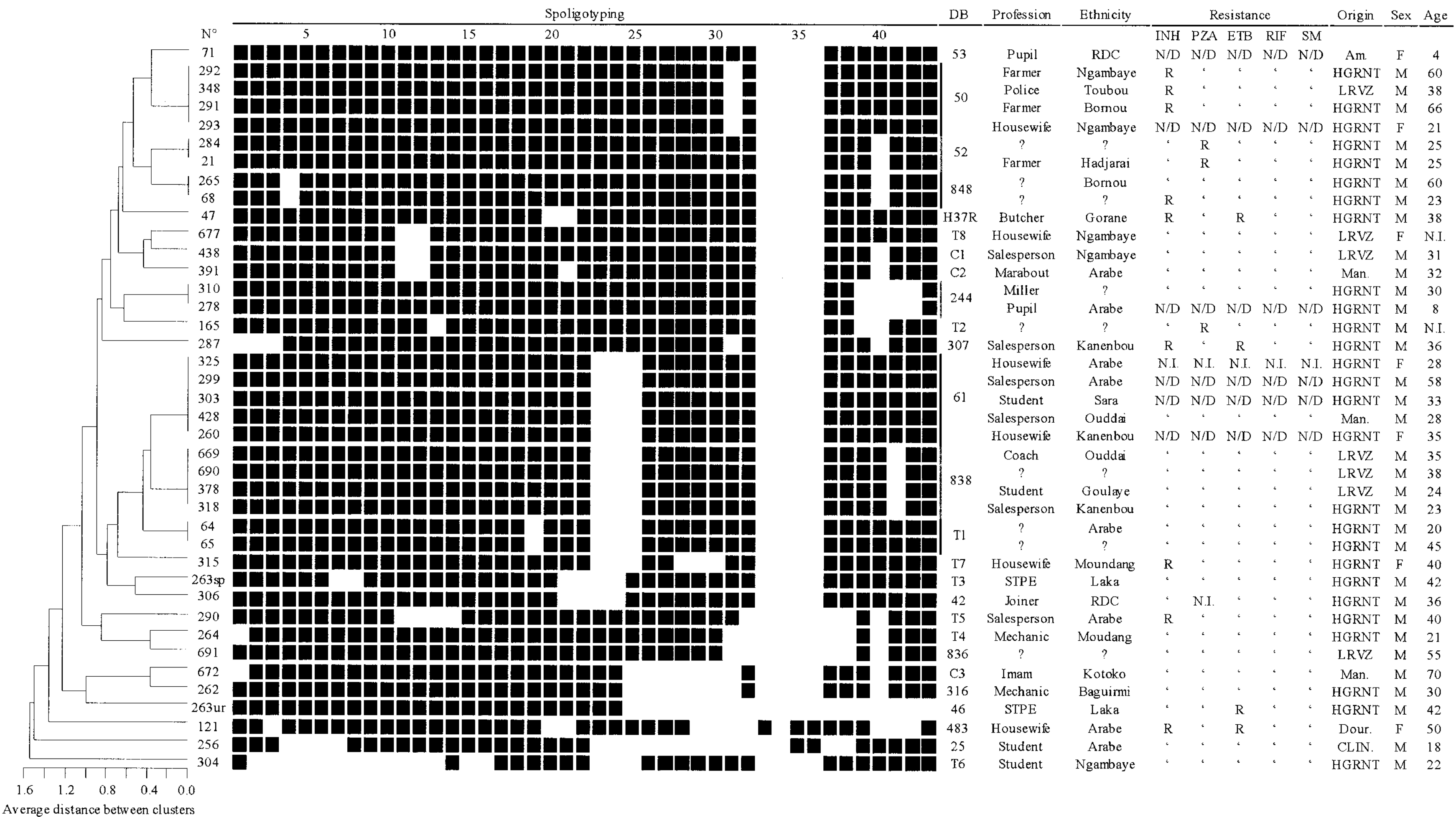

FIG. 1. Molecular characteristics and results of drug resistance testing of 40 isolates. Abbreviations and symbols: sp, sputum; ur, urine; T1 to T8, novel spoligotypes described in this study; C1 to C3, unique spoligotypes first described to occur in Cameroon; ?, unknown; DB, SpolDB3.0 (6). STPE, postman; RDC, Congo; PZA, pyrazinamide; ETB, ethambutol; SM, streptomycin; ′, susceptible; R, resistant; N.I., not identified; N/D, not done; Am., Am Dobak (Chad); HGRNT, hospital of N′Djaména; LRVZ, laboratory of N′Djaména; Man., Mandelia (Chad); Dour., Dourbali (Chad); CLIN, clinic of N′Djaména; F, female; M, male. Ages are given in years.

a separate analysis, the agar proportion method for INH, RIF, ethambutol, pyrazinamide, and streptomycin has been evaluated and compared to the MGIT method (4). Both methods were valid for the Chadian strains.

In total, drug susceptibility testing of 33 *M. tuberculosis* isolates showed that 20 (61%) were susceptible to all drugs, while 13 (39%) were resistant to at least one drug (Fig. 1). Resistance to isoniazid was the most frequent (nine patients [27%]; in three patients [9%] together with resistance to ethambutol). Resistance to ethambutol was observed with 4/30 isolates (12%) and to pyrazinamide with 3/30 isolates (10%). We found no resistance to rifampin and streptomycin in our sample. The level of resistance to INH is alarming (9/33; 27%) when we consider that INH is used as a first-line drug in Chad. (We did not evaluate the INH resistance testing at the concentration of 0.4 μg/ml, which is mandatory for INH-resistant isolates according to international standards, because the agar concentration showed such a good accordance.) Primary resistance to INH in other African countries is usually lower: 8% in Ethiopia (1), 12.5% in Equatorial Guinea (17), 12% in the West Province of Cameroon (11), and 7% in northern Nigeria (5). No strains were resistant to both streptomycin and rifampin. Resistance to these drugs is rather common in Africa, with exceptions such as Guinea-Bissau (resistance to INH only) (3) and Congo (no RIF-resistant isolates) (13). Interestingly, only one of nine Cameroon family isolates showed resistance to widely used antimycobacterial drugs. This indicates that drug resistance does not necessarily explain the high frequency of this family among Chadian isolates.

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