INTRODUCTION Tuberculosis (TB) remains a deadly infectious disease affecting millions of people worldwide; 95% of TB cases, with 98% of deaths, occur in developing countries (1). Approximately one-third of the world’s population is infected with tuberculosis, and 2 million people die of the disease every year (2). South Africa is a country with a high incidence of TB—600 cases per 100,000 population in 2005 (3), 550 cases per 100,000 population in 2003, and 718 cases per 100,000 population in 2004 (4,5). During the study period, the cases of drug resistance increased from 156 per 100,000 in 2004 to 177 per 100,000 in 2006 in Mpumalanga, and from 58 to 84 per 100,000 in Limpopo. However, Gauteng showed a high number of drug-resistant cases with an increase from 662 in 2004 to 794 per 100,000 in 2006 (3). World Health Organization recommends standardized TB treatment regimens based on short-course chemotherapy. The anti-TB drug regimen recommended for the treatment of new cases consists of IS6110.

Restriction Fragment Length Polymorphism Typing of Drug-resistant Mycobacterium tuberculosis Strains from Northeast South Africa

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ABSTRACT

Tuberculosis (TB) remains a deadly infectious disease affecting millions of people worldwide; 95% of TB cases, with 98% of death occur in developing countries. The situation in South Africa merits special attention. A total of 21,913 sputum specimens of suspected TB patients from three provinces of South Africa routinely submitted to the TB laboratory of Dr. George Mukhari (DGM) Hospital were assayed for Mycobacterium tuberculosis (MTB) growth and antibiotic susceptibility. The genetic diversity of 338 resistant strains were also studied. DNA isolated from the strains were restricted with Pvu II, transferred on to a nylon membrane and hybridized with a PCR-amplified horseradish peroxidase 245 bp IS6110 probe. Of the 338 resistant strains, 2.09% had less than 5 bands of IS6110, and 98% had 5 or more bands. Unique restriction fragment length polymorphism (RFLP) patterns were observed in 84.3% of the strains, showing their epidemiological independence, and 15.7% were grouped into 22 clusters.
Thirty-two strains (61.5%) from the 52 that clustered were from Mpumalanga, 16/52 (30.8%) from Gauteng, and 4/52 (9.6%) from Limpopo province. Clustering was not associated with age. However, strains from male patients in Mpumalanga were more likely to be clustered than strains from male patients in Limpopo and/or Gauteng province. The minimum estimate for the proportion of resistant TB that was due to transmission is 9.06% (52-22=30/331). Our results indicate that transmission of drug-resistant strains may contribute substantially to the emergence of drug-resistant tuberculosis in South Africa. Key words: Drug resistance; Epidemiology; IS6110; M. tuberculosis; PCR-RFLP; South Africa Green E et al. Typing of drug-resistant M. tuberculosis strains, using RFLP JHPN 2 a two-month administration of isoniazid (INH), rifampicin (rifampin, RIF), pyrazinamide (PZA), and ethambutol (EMB), followed by a continuation phase of INH/RIF and/or EMB for four months (2). However, this treatment is usually effective against MTB strains that have never been exposed to anti-TB drugs for more than 30 days (6) and against strains that do not possess drug-resistant mechanisms. For many years, Direct Observation of Treatment (DOT) has been promoted by the World Health Organization (WHO) as one of the five components of a wider strategy called Directly Observed Treatment, Short Course (DOTS) to tackle the resurgence of TB throughout the world (7). Direct observation by health workers while patients take their tablets aims to improve adherence to therapy and completion of treatment. Specific therapy for patients with drug-resistant tuberculosis is included in DOTS-plus. A surge in drug-resistant tuberculosis in several parts of the world requires effective implementation of the DOTS-plus strategy to prevent the occurrence of new multidrug-resistant (MDR) TB cases and to reduce transmission of MTB. Resistance to antibiotics in MTB occurs due to genomic mutations in certain genes, such as katG for INH resistance and rpoB for RIF resistance (8). Therefore, MTB will benefit from increased mutation rate. Unlike other pathogens with MDR pathotypes, such as transposable elements, plasmid-mediated mechanisms of resistance have not been reported in MTB (9,10). In recent years, treatment of TB has become complicated by increasing emergence of drug-resistant M. tuberculosis (DR-TB) (11). The proportion of MDR-TB strains in South Africa rose from 1.1% in 2004 to 1.9% in 2006 (3). Extremely drug-resistant (XDR) TB strains have also been reported in the country (12). Analysis of the spread and transmission of DR-TB strains, using molecular methods, has been reported (13). Techniques, such as restriction fragment length polymorphism (RFLP) and insertion sequence 6110 (IS6110), have been used in reliably differentiating M. tuberculosis isolates (14,15), and IS6110 has been suggested as a standard tool for characterization of isolates. IS6110 fingerprinting has also been used successfully to confirm laboratory cross-contaminations and to trace small-scale outbreak of TB and DR-TB in a large variety of settings (16). Although research on MTB transmission, using molecular techniques, has been conducted in the Western Cape (17), Kwazulu-Natal (18,19), Gauteng, and North West (20) province, similar information has not been recorded for DR-TB isolates from Gauteng, Limpopo, and Mpumalanga provinces. There is, therefore, a paucity of information on epidemiological data based on molecular methods addressing the transmission routes of DR-TB strains. This merits attention considering that the country has a high prevalence of HIV/AIDS, a confounding factor for TB resurgence. The aim of this study was to determine the genetic diversity of resistant MTB isolates, using IS6110 to delineate the dissemination of major phylogenetic clades of the organism in Gauteng, Limpopo, and Mpumalanga regions of South Africa. MATERIALS AND METHODS Study location and population The present study was conducted in the TB Laboratory of the DGM Hospital, Gauteng province of South Africa. This province showed a TB prevalence rate of 500/100,000 population in 2006 (21). From January
2004 to December 2006, sputum specimens of all patients (n=21,913) routinely sent for TB analysis to microbiology laboratory of DGM Hospital were included in the study. During the study period, we were aware of only three provinces that had TB diagnostic laboratories, which included Gauteng, Western Cape, and KwaZulu-Natal. We concentrated on DGM Hospital since it received specimens from other provinces, including Limpopo and Mpumalanga, without diagnostic centres. Patients’ characteristics The samples used were routinely collected from patients by hospitals and sent to DGM Hospital for TB diagnosis. Data on the patients, concerning type of specimens and standard demographic information (gender, age, and province), were registered. The study was approved by the Research and Ethics Committee of the University of Venda, South Africa. Informed consent was not obtained from the subjects.

Bacterial isolates All specimens were processed and cultured in the BacT/Alert 3D (BioMérieux, Durham, NC, USA) system. Positive cultures were stained with Ziehl Neelsen and confirmed with the AccuProbe DNA hybridization assay (Gen-Probe, USA) according to the manufacturer’s instructions. Isolates were then advanced for susceptibility testing and sequencing of katG and rpoB genes (22,23). The Green E et al. Typing of drug-resistant M. tuberculosis strains, using RFLP Volume 31 | Number 1 | March 2013 3 isolates of MTB analyzed in this study represent all available isolates obtained from patients of Limpopo, Mpumalanga, and Gauteng province, attending DGM Hospital between January 2004 and December 2006. The TB laboratory of DGM Hospital serves as one of the major TB laboratories situated in Gauteng province of South Africa. All the isolates were examined for their susceptibility to isoniazid (INH), ethambutol (EMB), streptomycin (SM), and rifampicin (RIF) following instructions of MGIT 960 system (MGITs; Becton Dickinson Microbiology systems, Sparks, MD, USA). Three hundred thirty-eight resistant strains of M. tuberculosis were obtained from 21,913 patients between January 2004 and December 2006. Of all the resistant strains obtained, 97 (28.7%) were from Gauteng, 32 (9.5%) from Limpopo, and 209 (61.8%) from Mpumalanga province. The provinces share borders with one another, with Mpumalanga situated in the Southeast of Limpopo and East of Gauteng, and Limpopo in the North of Gauteng. Extraction of DNA The MGIT 960 cultures were heat-inactivated at 80 °C for 1 hour before DNA extraction was performed in a biosafety level 2 laminar flow cabinet as previously described (22,24). Briefly, growth from the MGIT 960 system was suspended in 6 mL of DNA extraction buffer (5% monosodium glutamate, 50 mM Tris-HCl, pH 7.0 and 25 mM EDTA) in a sterile 50-mL polypropylene tube which contained approximately thirty 5-mm glass balls. The bacterial clumps were disrupted by vigorous shaking and vortexing. Five hundred microlitre of lysozyme (Amersham Biosciences, Greece) and 10 μL of RNAseA (Amersham Biosciences, Greece) were added to the tube. The contents of the tube were mixed by gentle inversion and then incubated at 37 °C for 2 hours. After incubation, 600 μL of 10×Proteinase K buffer and 150 μL of Proteinase K (Amersham Biosciences, Greece) were added. The sample was gently mixed (inverting the tube a few times) and then incubated overnight at 45 °C. Proteins were removed by phenol/chloroform and chloroform/isooamyl-alcohol extraction method (25). DNA was then precipitated with the addition of 600 μL of 3 M sodium acetate (pH 5.5) and 7 mL of cold (-20 °C) isopropanol. The precipitated DNA was washed with 1 mL of 70% ethanol for approximately 1 minute. DNA was then air-dried and resuspended in 30 μL in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Typing of M. tuberculosis strains, using IS6110 DNA was extracted using the phenol/chloroform method as described earlier (24,25). RFLP was performed using the standardized IS6110 technique as described previously (15). The extracted genomic DNA was restricted with Pvu II (20) in a reaction mix (final volume 30 mL) consisting of 3 μg of
genomic DNA, and 15 units of Pvu II in 3 μL of the prescribed restriction buffer (Amersham biosciences, Greece). The restriction mix was incubated overnight (±16 h) at 37 °C. At the end of digestion, the reaction was incubated at 65 °C for 10 minutes to inactivate any remaining enzyme activity. Restricted products were resolved on a 0.8% agarose at constant voltage of 40 in 1X TBE buffer for 24 hours. The probe used for hybridization was generated by PCR, using primers INS1 (5 CGT GAG GGC ATC GAG GTG GC 3) and INS2 (5/ GCG TAG GCG TCG GTG ACA AA 3/) labelled with horseradish peroxidase (ECLtm direct nucleic acid labelling and detection kit, Amersham, UK); hybridization and detection were carried out as per manufacturer’s instructions. We restricted the analysis to the isolates with 5 or more bands because isolates with few or no copies of the IS6110 element cannot be reliably classified by this method (26). IS6110 fingerprints were analyzed visually as described earlier (27). Recent transmission was considered likely if an isolate matched at least one other by identical or near-identical criteria. ‘Identical’ isolates were characterized by equal number of bands on gel electrophoresis, following digestion by restriction endonuclease; all such bands had to have matching molecular weights. ‘Near-identical’ isolates were characterized by difference of a single band (addition or loss of a single band). RFLP patterns were grouped from 1 to 20 based on the number of IS6110 band. A cluster was defined as a group of two or more patients with drug-resistant MTB strains whose fingerprints were identical with respect to both number and the size of all bands. We used the n-1 method to estimate continuing transmission. The method is based on the assumption that one case per cluster is due to reactivation and that this ‘index’ infectious case gives rise to other cases in the cluster either by infecting them directly or infecting a secondary case that then infects other members of the cluster. It calculates the number of recently-transmitted cases by summing within clusters after reducing each cluster-size by one giving the transmission dynamics (28). It is calculated by the following formula: Total number of strains in clusters [Number of clusters]/ Total number of strains