Research Article

Genotyping of Clinical Mycobacterium tuberculosis Isolates Based on IS6110 and MIRU-VNTR Polymorphisms

Anna Żaczek, Anna Brzostek, Arkadiusz Wojtasik, Jarosław Dziadek, and Anna Sajduda

1 Department of Biochemistry and Cell Biology, Faculty of Biology and Agriculture, University of Rzeszów, Ćwiklińskiej 2, 35-601 Rzeszów, Poland
2 Institute of Medical Biology, Polish Academy of Sciences, Lodowa 106, 93-232 Łódź, Poland
3 Proteon Pharmaceuticals, Tylna 3a, 90-364 Łódź, Poland
4 Department of Microbial Genetics, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland

Correspondence should be addressed to Anna Sajduda; asajduda@biol.uni.lodz.pl

Received 7 October 2013; Accepted 20 November 2013

1. Introduction

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis, one of the most dangerous human pathogens. In 2009 almost two million people worldwide died of TB [1]. Recently, molecular typing of M. tuberculosis has greatly improved our knowledge of TB epidemiology and allowed for a better control of this disease [2]. Identification of epidemiologically linked M. tuberculosis strains helps to reveal the source of infection, to trace the transmission routes of various strains, and to determine the risk factors for TB transmission in a community. Molecular epidemiology enables distinguishing between exogenous reinfection and endogenous reactivation, thus helping in a more effective elimination of TB from the population. In a laboratory, molecular methods can be used to identify cross-contamination [3–5].

Various genetic markers are currently used in molecular epidemiology of TB. A high degree of DNA polymorphism in M. tuberculosis strains is associated with repetitive DNA elements such as insertion sequences (IS) and short repetitive DNA sequences [2]. The insertion sequence IS6110 is especially useful in genotyping of M. tuberculosis [6]. IS6110 restriction fragment length polymorphism (RFLP) is one of the methods based on the variability of this element and is the current international typing standard in the
2. Materials and Methods

2.1. Bacterial Isolates. The 155 M. tuberculosis isolates, of 234 isolates previously studied [21], were available for the present analysis. They were obtained in 2005–2008 from 153 TB patients diagnosed at the Center for Lung Diseases Treatment and Rehabilitation in Łódź, Poland. In two patients, a second isolate was obtained in a time interval and those two replicative isolates were also included in the present study. The strains were cultured on Löwenstein-Jensen slants from sputum (n = 126), bronchial aspirate (n = 15), throat swab (n = 7), pleural fluid (n = 4), larynx swab (n = 1), gum pus (n = 1), and urine (n = 1). All the isolates were tested for their susceptibility to isoniazid (INH), streptomycin (SM), ethambutol (EMB), and rifampicin (RMP) using the BACTEC 460-TB system (Becton-Dickinson, Sparks, MD, USA). Only six (3.9%) strains were resistant to at least one anti-TB drug: INH (n = 3); SM (n = 1); INH and RMP (n = 1); INH, RMP and SM (n = 1).

Genomic DNA was extracted and purified from all the isolates using the protocol by van Embden et al. [7], recommended for the standard IS6110 RFLP typing. The concentration of DNA was measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) prior it was used in molecular typing.

2.2. Fast Ligation-Mediated PCR. FLiP was performed as originally described by Reisig et al. [16]. Briefly, the linker was synthesized by annealing of the oligonucleotides NLO (5'-GCATTGGATTCCACGTACGACTGCACG-3') and BA (5'-TGCAGUCGUCAGGUGGAA-3'). The oligonucleotides were added in equimolar amounts into 100 μL 1 × Gold Buffer (Invitrogen) and heated to 94 °C for 15 min, followed by three cycles with 58 °C for 10 min and 70 °C for 5 min. Next, the DNA samples were digested with 0.5 U/μl HhaI (Fermentas Life Technologies) and the restriction fragments were ligated to the linker (0.67 mM) using 0.05 U/μL T4 DNA ligase in 20 μL 1 × ligation buffer (Fermentas Life Technologies). Both reactions were performed simultaneously for 2 h at 25 °C. Five μL of the restriction-ligation mixture was used in amplification reaction. The PCR mix (50 μL) contained primers IS6110 (5'-TCGACTGGTCCACATCGCCGC-3') and Flip1 (5'-TTCATTTGATCCACGTACGACTGCACG-3') (12.5 pM each), 1.25 mM MgCl2, 0.8 mM deoxyribonucleoside triphosphates, 0.5 U uracil DNA glycosylase (UDG, Invitrogen), and 2.5 U AmpliTaq Gold DNA polymerase (Invitrogen). The reaction began with an incubation at 50 °C for 10 min to cut BA oligonucleotide of the linker with UDG. The cycling conditions consisted of an activation of AmpliTaq Gold DNA polymerase at 95 °C for 10 min, followed by 30 cycles with 30 s at 69 °C and 1 min at 72 °C, and a final extension for 7 min at 72 °C. Each experiment included negative (sterile ultrapure water) and positive (DNA of M. tuberculosis H37Rv) controls processed together with the test samples.

PCR products were resolved by 8% polyacrylamide gel electrophoresis. Banding patterns were visualized by ethidium bromide staining and photodocumented under UV light.

2.3. Genotype Analysis. The FLiP patterns were examined visually and then subject to computer-assisted analysis with BioNumerics 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity (percentage) of patterns was calculated by the unweighted pair group method with arithmetic averages (UPGMA) and Dice similarity coefficient. A cluster was defined as a group of at least two isolates showing 100% identical DNA fingerprints.

The Hunter-Gaston Discriminatory Index (HGDNI) [22] was used as a numerical index for the discriminatory power of the typing methods.
3. Results

Fast ligation-mediated PCR was applied in the present study to verify the usefulness of this method in differentiation of \textit{M. tuberculosis} strains. The FLiP fingerprint patterns of 155 strains tested were highly variable. The number of bands in the FLiP patterns ranged from 3 to 11, with fragments between 100 and 1000 bp. The majority, 130 (84%) of the patterns, contained 5–8 bands, with an average of 6 bands. Figure 1 shows an example of fingerprinting results for \textit{M. tuberculosis} strains obtained with FLiP method. FLiP typing proved to be highly reproducible. The reproducibility of FLiP was assessed by independent analysis of triplicate DNA samples of 16 randomly selected test strains. Also, DNA of \textit{M. tuberculosis} H37Rv was included as a positive control in each experiment. The FLiP patterns obtained for the reference strain and test strains in independent reactions were identical, confirming the reproducibility of this method (data not shown). However, weak (Figure 1, lane 7) and/or smear-like bands (Figure 1, lanes 2, 4, and 6) were sometimes visualized. Therefore, the unweighted pair group method with arithmetic averages (UPGMA) and Dice similarity coefficient based on manual indication of bands was used for the comparative analysis of FLiP patterns instead of a densitometry-based algorithm.

FLiP typing identified 119 distinct patterns among 155 tested strains (Figure 2). One hundred and one (65%) strains showed unique band patterns, and remaining 54 (35%) strains clustered into 18 groups containing 2–12 identical genetic profiles representing tested strains. The largest cluster was composed of 12 strains. Also, three larger clusters of strains were detected, each containing six, five, and three strains, respectively. Majority of the clusters (14 out 18) were made up of two strains each. Patterns detected in two strains isolated from the same patient at different times were identical and clustered together, further confirming stability and reproducibility of the FLiP profiles (Figure 2). The overall degree of strain differentiation generated by FLiP was 0.768 and it was higher in comparison with 0.697 observed for the same set of strains when characterized by 15-locus MIRU-VNTR typing.

Previously performed, MIRU-VNTR analysis [21] identified 108 distinct profiles among the 155 strains tested. Eighty-one (52%) strains were unique, and 74 (48%) strains were grouped in 27 clusters including 2–11 isolates. Eleven strains were grouped into the largest cluster with identical MIRU-VNTR patterns. Three large clusters of strains were also identified, each containing seven, six, and four strains, respectively. The remaining 23 clusters were composed of two strains each. As in FLiP, the repetitive isolates from two patients were identical to the first isolate also in MIRU-VNTR typing. Clustering results from both methods were compared to determine their discriminatory power. FLiP gave resolving power only slightly higher than the 15-locus MIRU-VNTR analysis (HGDI = 0.991 and 0.990, resp.). However, detailed comparison of the clusters defined by each of the methods revealed a more apparent difference in the discriminatory abilities that favored FLiP. As shown in Table 1, 13 (48%) of the 27 MIRU-VNTR clusters included strains with multiple FLiP patterns, whereas only 4 (22%) out of the 18 FLiP clusters could be subdivided by MIRU-VNTR analysis.

The simultaneous use of both methods identified 42 isolates that clustered in 15 combined FLiP/MIRU-VNTR groups. Previously obtained IS6110 RFLP data [21] revealed one unique pattern in each of three combined clusters consisting of seven, four, and three strains, respectively. The differences in IS6110 RFLP patterns within the three subdivided FLiP/MIRU-VNTR clusters were, however, not significant and limited only to the presence of one or two additional IS6110 hybridizing bands (data not shown).

4. Discussion and Conclusion

FLiP is a DNA typing method for \textit{M. tuberculosis} complex strains described by Reisig et al. in 2005 [16]. It is based on the original mixed-linker approach that used one primer specific for IS6110 and a second primer complementary to a linker ligated to the \textit{HhaI} restriction fragments of genomic DNA in a PCR amplification. One strand of the linker molecule contained uracil instead of thymidine, and it was split by UDG assuring specificity of the reaction [17]. The mixed-linker method was successfully applied to outbreak investigations and population-based studies [17, 18, 23]. However, it requires reamplification that increases the risk of cross-contamination and multiple hands-on steps. To overcome those limitations, Reisig et al. constructed new linker oligonucleotides. That modification enabled specific amplification of RFLP fragments carrying IS6110 after a single PCR step, following simultaneous restriction-ligation reactions, resulting in a simplified and faster typing of \textit{M. tuberculosis} strains [16]. Kremer et al., in an extensive interlaboratory study comparing nine PCR-based assays, concluded that MIRU-VNTR and FLiP methods are both rapid, highly reliable, and discriminative epidemiological typing tools for \textit{M. tuberculosis} [18]. However, in spite of its potential to differentiate between strains, FLiP has not been tested in molecular analyses since its publication.

To address this lack of data, we previously used FLiP to estimate molecular relationships among small sets of clinical \textit{M. tuberculosis} isolates [19, 20]. In this study, we extend
our preliminary observations and report a molecular characterization of 155 *M. tuberculosis* strains from Łódź, Poland, through genotyping by FLiP in comparison with previous results of 15-locus MIRU-VNTR analysis [21]. In accordance with our earlier reports [19, 20], FLiP patterns obtained in the present study consisted of 3–11 DNA fragments, with an average of 6 bands. This is slightly lower compared to 0–16 bands (8 average) described by Reisig et al. [16]. These authors did not comment on concordance between the numbers of IS6110 copies detected by FLiP and IS6110
Table 1: Comparison of clustering in M. tuberculosis strains by the use of 15-locus MIRU-VNTR and FLiP typing methods.

| Pattern (no. of strains) | MIRU-VNTR | FLiP pattern (no. of strains) | Pattern (no. of strains) | MIRU-VNTR | FLiP pattern (no. of strains) |
|-------------------------|-----------|-------------------------------|-------------------------|-----------|-------------------------------|
| 1 (11)                  | 45353133243437 | 1 (6), 2 (1), 3 (1), 4 (2), 5 (1), 6 (1) | 16 (2)                  | 42353332242325 | 30 (1), 31 (1) |
| 2 (7)                   | 482132544334228 | 7 (12)^b | 18 (2)                  | 44343334242225 | 33 (2) |
| 3 (6)                   | 45353333443335 | 8 (3), 9 (1), 10 (1), 11 (1) | 19 (2)                  | 42343434242526 | 34 (1), 35 (1) |
| 4 (4)                   | 343541333443446 | 12 (5) | 21 (2)                  | 4323534242325 | 37 (2) |
| 5 (2)                   | 44353333443437 | 13 (2) | 22 (2)^d | 43153532243222 | 38 (2)^d |
| 6 (2)                   | 45353333443337 | 14 (2) | 23 (2)                  | 44344333443545 | 39 (2) |
| 7 (2)                   | 43243333443437 | 15 (1), 16 (1) | 24 (2)                  | 3121323443138 | 40 (1), 41 (1) |
| 8 (2)                   | 463643334434637 | 17 (1), 18 (1) | 25 (2)                  | 41021345444326 | 42 (2) |
| 9 (2)                   | 43353333443437 | 19 (1), 20 (1) | 26 (2)                  | 49213454443138 | 43 (1), 44 (1) |
| 10 (2)^c                | 442431542132437 | 21 (2)^c | 27 (2)                  | 48253244343228 | 7 (2)^b |
| 11 (2)                  | 45353133243237 | 7 (1)^b, 22 (1) | 28 (1), 29 (1) | 45353333443345 | 45 (2) |
| 12 (2)                  | 45353333443437 | 23 (2) | 45353333443342 |
| 13 (2)                  | 46363333443235 | 24 (1), 25 (1) | 30 (1), 31 (1) | 45353333475347 | 46 (2) |
| 14 (2)                  | 423432342242425 | 26 (1), 27 (1) | 4534333463248 |
| 15 (2)                  | 432532342242425 | 28 (1), 29 (1) | | | |

^a Number of copies > 9 per locus is underlined.

^b Members (n = 12) of the same cluster (FLiP pattern 7).

^c,d Sequential isolates from two patients.

RFLP. However, we previously noticed 1–8 bands less in 90% of FLiP patterns in comparison with IS6110 RFLP profiles of the respective strains [20]. Also, Prod’hom et al. reported that the number of bands generated in ligation-mediated PCR, a DNA typing technique related to mixed-linker amplification, was equal to or lower than IS6110 copy number in the strains tested [24]. These results indicate that the number of bands in the FLiP DNA fingerprints may not necessarily reflect the number of IS6110 copies in M. tuberculosis strains and rather should not be identified with them. Some limitation of FLiP, inherent to banding profiles-based methods, could be occasional appearance of weak and/or smear-like bands within a pattern. However, despite this potential difficulty, FLiP still proved to be fully reproducible confirming previous observations [18, 20].

FLiP typing revealed a high degree of polymorphism among the 155 strains investigated here confirming previous data of Kremer et al. [18], although its discriminatory power was somewhat lower in the present study (HGDI = 0.991 versus 0.994). The difference was most probably due to various geographical origins of the 90 M. tuberculosis strains tested earlier that resulted in lower degree of clustering compared to the present study on the strains originating from a single town only. Nevertheless, FLiP was still more discriminative than 15-locus MIRU-VNTR typing (HGDI = 0.990). On the other hand, Kremer et al. observed slightly lower discriminatory potential of FLiP compared to MIRU-VNTR analysis based on 12 loci (HGDI = 0.995) [18].

Strain resolution by MIRU-VNTR typing approaches that of the reference method but varies according to the loci analyzed and between strain families [18, 25–29]. Moreover, the exclusive use of this method leads to misinterpretations of epidemiological links among M. tuberculosis isolates [29, 30]. Methods based on IS6110 and methods based on MIRU-VNTR polymorphisms detect changes in different regions of the chromosome (mobile versus core); therefore clustering generated by the two types of methods does not have to be identical. In the present study, only 7% (3/42) of the strains clustered both by MIRU-VNTR and FLiP methods could be subdivided by the reference IS6110 RFLP. These results further confirm a high discriminatory ability of FLiP and its usefulness as a secondary typing technique for verification of MIRU-VNTR clusters.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by Grants from the National Science Centre (NCN, Project no. N N302 111338) and the European Regional Development Fund within the Operational Program Innovative Economy (Project no. POIG.01.01.02-10-107/09).

References

[1] World Health Organization, Global Tuberculosis Control: WHO Report 2010, WHO, Geneva, Switzerland, 2010.

[2] B. Mathema, N. E. Kurepina, P. J. Bifani, and B. N. Kreiswirth, “Molecular epidemiology of tuberculosis: current insights,” Clinical Microbiology Reviews, vol. 19, no. 4, pp. 658–685, 2006.
[3] S. Narayanan, "Molecular epidemiology of tuberculosis," *Indian Journal of Medical Research*, vol. 120, no. 4, pp. 233–247, 2004.

[4] C. Allix-Béguec, M. Fauville-Dufaux, and P. Supply, "Three-years population-based evaluation of standardized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat," *Journal of Clinical Microbiology*, vol. 46, no. 4, pp. 1398–1406, 2008.

[5] A. Shabbeer, C. Ozczagar, B. Yener, and K. P. Bennett, "Web tools for molecular epidemiology of tuberculosis," *Infection, Genetics and Evolution*, vol. 12, no. 4, pp. 767–781, 2012.

[6] D. Thierry, M. D. Cave, K. D. Eisenach et al., "IS6110, an IS-like element of *Mycobacterium tuberculosis* complex," *Nucleic Acids Research*, vol. 18, no. 1, pp. 188–193, 1990.

[7] J. D. A. van Embden, M. D. Cave, J. T. Crawford et al., "Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology," *Journal of Clinical Microbiology*, vol. 31, no. 2, pp. 406–409, 1993.

[8] M. A. Behr and S. Mostowy, "Molecular tools for typing and branding the tubercle bacillus," *Current Molecular Medicine*, vol. 7, no. 3, pp. 309–317, 2007.

[9] J. Kamerbeek, L. Schouls, A. Kolk et al., "Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology," *Journal of Clinical Microbiology*, vol. 35, no. 4, pp. 907–914, 1997.

[10] L. Qian, J. D. A. van Embden, A. G. M. van der Zanden, E. F. Weltevreden, H. Duanmu, and J. T. Douglas, "Retrospective analysis of the Beijing family of *Mycobacterium tuberculosis* in preserved lung tissues," *Journal of Clinical Microbiology*, vol. 37, no. 2, pp. 471–474, 1999.

[11] P. Supply, C. Allix, S. Lesjean et al., "Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*," *Journal of Clinical Microbiology*, vol. 44, no. 12, pp. 4498–4510, 2006.

[12] A. Sajduda, J. Dziadek, R. Kotłowski, and F. Portaels, "Evaluation of multiple genetic markers for typing drug-resistant *Mycobacterium tuberculosis* strains from Poland," *Diagnostic Microbiology and Infectious Disease*, vol. 55, no. 1, pp. 59–64, 2006.

[13] J. L. de Beer, J. van Ingen, G. de Vries et al., "Comparative study of IS6110 restriction fragment length polymorphism and variable-number tandem-repeat typing of *Mycobacterium tuberculosis* isolates in the Netherlands, based on a 5-year nationwide study," *Journal of Clinical Microbiology*, vol. 51, no. 4, pp. 1993–1998, 2013.

[14] Q. -F. Liang, Y. Pang, Q. -Y. Chen et al., "Genetic profile of tuberculosis among the migrant population in Fujian Province, China," *International Journal of Tuberculosis and Lung Disease*, vol. 17, no. 5, pp. 655–661, 2013.

[15] A. Thong-On, N. Smittipat, T. Juthayothin et al., "Variable-number tandem repeats typing of *Mycobacterium tuberculosis* isolates with low copy numbers of IS6110 in Thailand," *Tuberculosis*, vol. 90, no. 1, pp. 9–15, 2010.

[16] F. Reisig, K. Kremer, B. Amthor, D. van Soolingen, and W. H. Haas, "Fast ligation-mediated PCR, a fast and reliable method for IS6110-based typing of *Mycobacterium tuberculosis* complex," *Journal of Clinical Microbiology*, vol. 43, no. 11, pp. 5622–5627, 2005.

[17] W. H. Haas, W. R. Butler, C. L. Woodley, and J. T. Crawford, "Mixed-linker polymerase chain reaction: a new method for rapid fingerprinting of isolates of the *Mycobacterium tuberculosis* complex," *Journal of Clinical Microbiology*, vol. 31, no. 5, pp. 1293–1298, 1993.

[18] K. Kremer, C. Arnold, A. Cataldi et al., "Discriminatory power and reproducibility of novel DNA typing methods for *Mycobacterium tuberculosis* complex strains," *Journal of Clinical Microbiology*, vol. 43, no. 11, pp. 5628–5638, 2005.

[19] A. Żaczek, A. Brzostek, A. Górna, A. Sajduda, A. Wojtasik, and J. Dziadek, "Application of FLiP method for differentiation of *Mycobacterium tuberculosis* strains in comparison to commonly used methods, spoligotyping and MIRU-VNTR typing," *Polish Journal of Microbiology*, vol. 62, no. 1, pp. 73–76, 2013.

[20] A. Żaczek, M. Ziółkiewicz, A. Wojtasik, J. Dziadek, and A. Sajduda, "IS6110-based differentiation of *Mycobacterium tuberculosis* strains," *Polish Journal of Microbiology*, vol. 62, no. 2, pp. 201–204, 2013.

[21] M. Krawczyk, A. Brzostek, A. Gorna et al., "Epidemiological analysis of *Mycobacterium tuberculosis* strains isolated in Lodz, Poland," *International Journal of Tuberculosis and Lung Disease*, vol. 15, no. 9, pp. 1252–1258, 2011.

[22] P. R. Hunter and M. A. Gaston, "Numerical index of the discriminatory ability of typing systems: an application of Simpson’s index of diversity," *Journal of Clinical Microbiology*, vol. 26, no. 11, pp. 2465–2466, 1988.

[23] M. Malaghini, S. R. Brockelt, M. Burger, A. Kritski, and V. Thomaz-Soccol, "Molecular characterization of *Mycobacterium tuberculosis* isolated in the State of Paraná in southern Brazil," *Tuberculosis*, vol. 89, no. 1, pp. 101–105, 2009.

[24] G. Prod’Horn, C. Guilhot, M. C. Gutierrez, A. Varnerot, B. Gicquel, and V. Vincent, "Rapid discrimination of *Mycobacterium tuberculosis* complex strains by ligation-mediated PCR fingerprint analysis," *Journal of Clinical Microbiology*, vol. 35, no. 12, pp. 3331–3334, 1997.

[25] R. X. Liu, Q. Z. Li, L. L. Xing, Z. Peng, C. M. Zhu, and Z. H. Yang, "Genotyping of clinical *Mycobacterium tuberculosis* isolates based on eight loci of MIRU-VNTR," *International Journal of Tuberculosis and Lung Disease*, vol. 17, no. 2, pp. 243–245, 2013.

[26] M. M. Sankar, J. Singh, S. C. A. Diana, and S. Singh, "Molecular characterization of *Mycobacterium tuberculosis* isolates from North Indian patients with extrapulmonary tuberculosis," *Tuberculosis*, vol. 93, no. 1, pp. 75–83, 2013.

[27] A. Chatterjee and N. Mistry, "MIRU–VNTR profiles of three major *Mycobacterium tuberculosis* spoligotypes found in western India," *Tuberculosis*, vol. 93, no. 2, pp. 250–256, 2013.

[28] B. J. Marais, C. K. Mlambo, N. Rastogi et al., "Epidemic spread of multidrug-resistant tuberculosis in Johannesburg, South Africa," *Journal of Clinical Microbiology*, vol. 51, no. 6, pp. 1818–1825, 2013.

[29] R. Sloat, M. W. Borgdorff, J. L. de Beer, J. van Ingen, P. Supply, and D. van Soolingen, "Clustering of tuberculosis cases based on variable-number tandem-repeat typing in relation to the population structure of *Mycobacterium tuberculosis* in the Netherlands," *Journal of Clinical Microbiology*, vol. 51, no. 7, pp. 2427–2431, 2013.

[30] L. S. Cowan, L. Diem, T. Monson et al., "Evaluation of a two-step approach for large-scale, prospective genotyping of *Mycobacterium tuberculosis* isolates in the United States," *Journal of Clinical Microbiology*, vol. 43, no. 2, pp. 688–695, 2005.