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Molecular characterization of Sardinian *Mycobacterium tuberculosis* isolates by IS6110 restriction fragment length polymorphism, MIRU-VNTR and rep-PCR

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SUMMARY

An evaluation of the utility of rep PCR typing compared to the 15 loci discriminatory set of MIRU-VNTR was undertaken. Twenty-nine isolates of *Mycobacterium tuberculosis* from patients were examined. Genomic DNA was extracted from the isolates by standard method. The number of copies of tandem repeats of the 15 MIRU-VNTR loci was determined by PCR amplification and agarose gel electrophoresis of the amplicons. *M. tuberculosis* outbreak-related strains were distinguished from other isolates. MIRU-VNTR typing identified 4 major clusters of strains. The same isolates clustered together after RFLP typing, but rep-PCR identified only 3 of them. The concordance between RFLP and MIRU-VNTR typing was complete, with the exception of two isolates with identical RFLP patterns that differed in the number of tandem repeat copies at two MIRU-VNTR alleles. A further isolate, even sharing the same RFLP pattern, differed by one repeat from the rest of its cluster. We also tested the use of an automated rep-PCR for clinical laboratory applications but it failed to identify the link between two pairs of epidemiologically related strains clustered by the other 2 techniques. For superior discrimination, ease of comparison of results and lower cost, MIRU-VNTR typing should be the favored PCR-based typing tool.

KEY WORDS: IS6110-RFLP, MIRU-VNTR, Rep-PCR, *Mycobacterium tuberculosis*

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INTRODUCTION

Mycobacterium tuberculosis is a successful worldwide human pathogen responsible of killing about 3 million people every year (WHO Report, 2006), it has been estimated that approximately one-third of the world population has latent infections (Dye *et al.*, 1999). During the last 2 decades the introduction of molecular tools has enabled us to better understand and track tuberculosis (TB) transmission and epidemiology. TB molecular epidemiology exploits selected bacterial DNA targets to serve as markers for *M. tuberculosis* strains (Barnes and Cave, 2003).The most polymorphic regions targeted include: insertion sequences (IS), direct repeats (DR) and tandem repeats. As a result every strain produces specific genetic profiles called fingerprinting. Identical strain fingerprints are called clusters and are usually associated with recent transmission, while strains presenting unique fingerprint profile suggest remote transmission or infection acquired in the past.

The most established typing method, IS*6110*-based restriction fragment length polymorphism (RFLP), exploits the variable presence and copy number (0-.25) of the IS*6110* transposable element among *M. tuberculosis* complex (MTC) strains. IS*6110* RFLP has been used in many epidemiological studies to detect outbreaks and

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track TB transmission (Alland e*t al.*, 1994), to uncover laboratory cross-contamination (Braden *et al.*, 1997) and to distinguish exogenous reinfection from endogenous reactivation in relapse cases. Although this method has been widely used as the gold standard method for years (Kremer *et al.*, 1999), it is labor-intensive, requires well-grown cultures, and cannot type strains which harbor few IS*6110* copy numbers (≥5). In addition this method generates data as an analogue band pattern, difficult to reproduce and compare among different laboratories. Those drawbacks have prompted researchers to develop alternative typing methods that can give faster results and can be easily compared.

A rapid PCR-based typing tool like MIRU-VNTR (mycobacterial interspersed repetitive units- variable numbers of tandem repeats) enables DNA fingerprints to be obtained from a small number of bacteria or even directly from clinical samples increasing the speed of identification of the organism (Mazars *et al.*, 2001). Various studies using mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTRs) based typing show that the discriminatory power of this method is close to that of the IS*6110* based method (Hawkey *et al*., 2003); and produces more distinct patterns in case of low IS*6110* copy number isolates (Cowan *et al.*, 2002; Dye *et al.*, 1999).

This genotyping method has been optimized and proposed for standardization (Supply *et al.*, 2006). This study used the discriminatory set of 15 loci, suitable for epidemiological study, to type 29 patient isolates of *M. tuberculosis*. The 15-digit numerical code results obtained for each sample were compared to the fingerprint given by IS*6110* RFLP and to that obtained with another PCR-based typing method the rep-PCR (repetitive-unit-sequence-based PCR) to establish the feasibility and discriminatory power of those methods.

Rep-PCR requires small amounts of *M. tuberculosis* culture material and produces high-resolution DNA fingerprints, thanks to an automated and standardized system: the DiversiLab system (MO Bio Laboratories, Solana Beach, Calif.) A description of this automated rep-PCR technology and its performance characteristics have been recently published (Healy *et al.*, 2005).

This molecular tool was initially developed for DNA fingerprinting of nosocomial pathogens such as *S. aureus* (Del Vecchio *et al.*, 1995) and *S. pneumonia* (Versalovic *et al.*, 1993), and then adapted to mycobacteria (Cangelosi *et al.*, 2004). This system has been used for outbreak investigation (Freeman *et al.*, 2005) and to type *M. tuberculosis* and *M. avium complex* isolates (Cangelosi *et al.*, 2004).

Specific regions, located between noncoding repetitive sequences of the bacterial genome, are amplified with the rep-PCR reagent kit, the resulting DNA amplicons are then separated on microfluid cheaps by the Agilent 2100 bioanalyzer. The software analyzes results (fluorescent intensity and migration time) and generates a dendrogram, a gel-like image and a scatter plot that are used for comparative analysis. Few studies have been carried out in Sardinia to characterize *M. tuberculosis* genotypes, the data available are limited and rely only on IS*6110* based RFLP typing (Sechi *et al*., 1996). This lack of information and the fact that Sardinia being an island represents an optimal setting for epidemiological studies, prompted us to investigate further.

The objective of this preliminary study was to assess the reliability of the DiversiLab system and MIRU-VNTR in determining the relatedness of *M tuberculosis* strains in Sardinia. In order to establish this, the results achieved were compared with the fingerprinting obtained by the IS*6110* based-RFLP typing.

MATERIALS AND METHODS

Mycobacterial isolates

M. tuberculosis clinical isolates from different parts of the Sardinian region were collected by the Diagnostic Laboratory of Tuberculosis, Department of Biomedical Science, Sassari, Italy. Bacteria were grown on Middlebrook 7H10 agar with oleate-albumin-dextrose-catalase enrichment.

A total of 29 *M. tuberculosis* clinical isolates, identified previously by IS*6110* nested PCR and biochemical methods (Vestal A.L. 1977), were characterized by IS*6110*-based restriction fragment length polymorphism (IS*6110*-RFLP) analysis, mycobacterial interspersed repetitive unit-variable numbers of tandem repeats (MIRU-VNTR) analysis, repetitive sequence-based PCR (rep-PCR) and with the Diversilab System (Biomiereux).

Sixteen out of 29 patients presented social links or a family relationship.

DNA extraction
DNA was extracted from *M. tuberculosis* cultures according to standard protocols (van Soolingen *et al.,* 1991). For rep-PCR analysis, DNA was extracted from a 10 µl loop of each Mycobacterium colony using the Ultra Clean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, Calif.). When necessary, the DNA was stored at -20°C until required.

MIRU-VNTR analysis
MIRU-VNTR genotyping was performed by amplifying the15 MIRU loci discriminatory subset for epidemiological study. The PCR primers and amplification conditions described by Supply. (Supply *et al*., 2006), were followed. The numbers of tandem-repeat units were determined by estimating the sizes of the amplicons on 2% agarose gels. H37Rv DNA and sterile water were used respectively as positive and negative controls. The 15- digit numerical code results obtained were entered in the database freely available online at http://www. MIRU-VNTRplus.org.

IS*6110* RFLP typing
Genomic DNA was digested with *Pvu*II, separated by agarose gel electrophoresis and transferred by Southern blotting to a nylon membrane according to the international standard protocol for *M. tuberculosis* (van Embden *et al*., 1993). DNA fingerprinting was performed by hybridization overnight with the IS*6110* peroxidase-labeled probe using an Enhanced chemiluminescence method (ECL Amersham, Biosciences, Chalfont St. Giles, United Kingdom).

Cluster analysis
IS*6110* RFLP results were read by 3 independent readers, and genotypes were assigned by consensus. A cluster was defined as a series of isolates that had 100% IS*6110* fingerprint identity. Band tolerance levels were set at 1%.

Diversilab v3.3
For rep-PCR analysis, genomic DNA was amplified using the DiversiLab Mycobacterium typing kit (Bacterial Barcodes, Inc., Solana Beach, Calif.). H37Rv was used as control. The amplicons of various sizes and fluorescent intensities were separated and detected using a microfluidics chip with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The Web-based Diversilab software (version 2.1.66) was used for further analysis.
It automatically compares the rep-PCR-based DNA fingerprints of unknown isolates through the Pearson correlation coefficient and unweighted pair group method with arithmetic mean. The report generated by the DiversiLab System contained a dendrogram and a scatter plot for sample comparison. A cut-off value of 95% similarity (Barnes and Cave, 2003) was used to establish strain identity.

RESULTS

IS*6110*-RFLP
The IS*6110* fingerprinting subdivided 29 *M. tuberculosis* isolates into 13 unique types and 4 clusters. The range of IS*6110* copies among isolates varied from 6-18. Based on the copy number, *M. tuberculosis* isolates were classified into 3 groups:
1) low copy number (6);
2) intermediate copy number (8-9) and
3) (high copy number (15-18), however, copy number higher than 18 was not observed in any of the isolates studied.

Thirty per cent of the isolates studied showed a high copy number of IS*6110*, 52% showed intermediate copy number, 18% showed low copy number, isolates presenting less than 6 copy number were not included in the analysis. For the 16 isolates clustered in 4 groups, we also found social links and blood relations, which can further justify the clustering (Table 1).
The discriminatory power was calculated by using Simpson - index of diversity and its value was found to be approximately 1.0.

MIRU-VNTR
The majority of the 29 isolates exhibited distinct MIRU-VNTR genotypes by amplifying with primers for 15 MIRU loci (Supply *et al*., 2006). MIRU locus 4052 was found to be the most discriminatory locus with 8 alleles. A minimum of 3 and a maximum of 11 repeats of this locus were found in our study and allele number 4 was the most common. Other loci were less variable with

TABLE 1 - *Clusters of isolates, ties between the members and typing tools results.*

Cluster	*Strains' ID*	*Social-family relationships*	*RFLP*	*MIRU-VNTR*	*Rep-PCR*
a)	475/06 507/06 523/06 547/06	Schoolmates	Same copy Number and molecular weight	Only 2 isolates differed in the number of repeats at 2 loci	More than 95% similarity
b)	71/01 571/99 143/06 or134/06 181/06 or 214/06 236/06 or 266/06	Acquaintances who share the same car to go to work	Same copy Number and molecular weight	Only1isolate differed in the number of repeats at 1 locus	More than 95% similarity
c)	697/06 819/06 826/06	Mother son and daughter	Same copy Number and molecular weight	Same 15 digit code	More than 95% similarity
d)	400/07 441/07 + 520/06 118/07	Brother and sister plus 2 coworkers from Banari	Same copy Number and molecular weight for all members	Same 15 digit code for all members	More than 95% similarity for brother and sister, but not similar to the 2 clustered coworkers

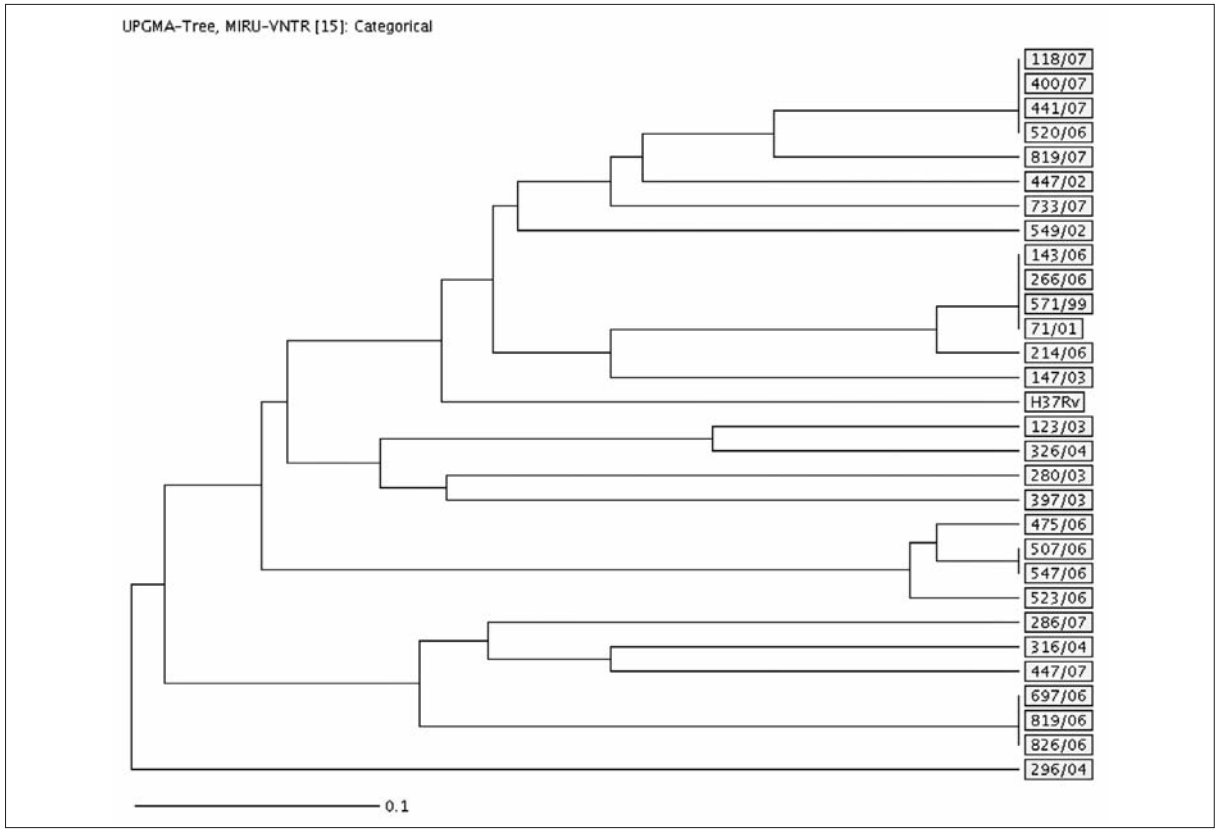

FIGURE 1 - *Dendrogram constructed based on MIRU-VNTR genotypes using the UPGMA method.*

7 alleles in locus 802, 5 alleles in locus 960, 4 alleles in loci 424, 3690, 1955, 2163 and 2996. In locus 960, allele 2 was the most common, present in 14 isolates.
Similarly, allele 2 was the most common in locus 424, found in 16 isolates. Loci 2165, 2401, 1644 and 3192 exhibited 3 alleles while loci 577 and 4156 only 2. Least variable was MIRU locus 4 alias 580, in fact allele 2 was found to be commonly present in all 29 isolates.
The dendogram (Figure 1) generated by using the UPGMA algorithm is based on MIRU-VNTR patterns which describe genetic relatedness among the 29 isolates. Sixteen isolates were closely related and they clustered in 4 different groups. The concordance between RFLP and MIRU-VNTR typing was complete with the exception of two isolates in cluster a) with identical RFLP patterns, that differed in the number of tandem repeat copies at two MIRU-VNTR alleles (523/06 locus 2996 and 475/06 locus 802). A further isolate of cluster b) (214/06) had one repeat more at locus 1644 compared to the rest of the cluster, but identical RFLP fingerprint.
After entering our strains in the MIRU-VNTR*plus* database, we carried out a best matches analysis against the 186 reference strains collection representative of the main principal MTBC lineages. A total match was found for a single strain. We then performed the analysis without using the database content, just comparing our strains one against the other to generate a dendrogram which describes the genetic relatedness of the 29 isolates under study.

Analysis of *M. tuberculosis* isolates by automated rep-PCR

Isolates were analyzed with the DiversiLab Mycobacterium typing kit (Bacterial Barcodes, Inc., Solana Beach, CA, USA). The dendrogram and virtual gel images indicate strain level grouping of the 29 *M. tuberculosis* isolates (Figure 2). Rep-PCR also identified 4 main clusters: the first 3 being the same identified by RFLP and MIRU-VNTR typing and a rather different fourth one. To this cluster belong 2 isolates of cluster d) the brother and sister from SS (SS400/07; SS441/07) and 3 isolates from NU and OR (NU447/02; NU549/02; OR147/03). All the other samples clustered separately from each other, as observed after applying the other 2 typing tools. After repeating the analysis three times we had discordant results for the 2 coworkers from Banari. The first time the 2 strains had 76% similarity (different), the second and third they turned out to be similar with strain 520/06 deriving from strain 118/07.

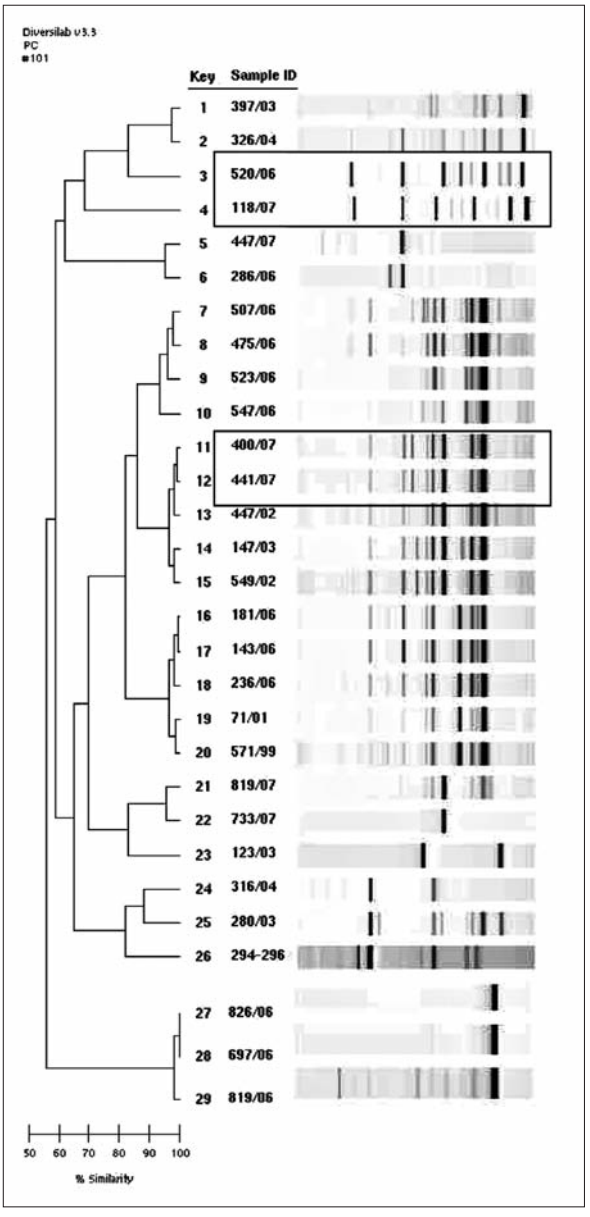

FIGURE 2 - *Rep-PCR-generated dendrogram and virtual gel images representing rep-PCR fingerprint patterns of 29 M tuberculosis isolates from Sardinia. An SI cutoff of 95% was used for interpretation of relatedness. The four isolates, which rep-PCR fail to cluster are depicted by rectangle.*

DISCUSSION

Epidemiological studies have helped shed light on the transmission dynamics and spread of *M. tuberculosis* strains. But the most widely used typing tool IS*6110*-RFLP is unable to give us results in "real time", so it quite often ends up giving results once outbreaks are already established. For local outbreaks and investigations of laboratory cross-contamination where speed is of the utmost importance suspect strains should be initially investigated using a PCR-based method. For this reason we performed both Rep-PCR and MIRU-VNTR typing for a total of 29 *Mycobacterium tuberculosis* patient isolates from the Sardinian region. MIRU accurately identified 4 clusters which were also defined by IS*6110*-based RFLP and three of these were confirmed by Rep-PCR. Both MIRU-VNTR and RFLP identified 16 linked isolates involved in cross-infection among groups of 3, 4 and 5 people. To note, even being isolated 6 and 7 years before the other members of cluster b), isolates 71/01 and 571/99 have the same RFLP pattern but present a polymorphism at locus 1644, suggesting a higher rate of evolution at this locus compared to that of IS*6110*- RFLP patterns. For all the members of cluster b) Rep-PCR found a very high percentage of similarity with a maximum of 99.2% for strains 143/06 and 214/06. In case of cluster a) the concordance among the 3 techniques was 100%, with MIRU-VNTR typing that was even able to further discriminate this isolate due to the number of repeats at 2 loci (802,2996).

The 3 techniques gave identical fingerprints for the family members of cluster c). The same, as might be expected, was found for the 2 family members of cluster d) but in this case MIRU-VNTR typing and RFLP linked these (400/07; 441/07) with other cases (520/06; 118/07) leading to the discovery of an unsuspected chain of transmission. Rep-PCR failed to identify this association and gave ambiguous results for the two coworkers, but found a high percentage of similarity between the 2 family members and 3 other isolates (447/02; 549/02; 147/03). At the moment we know only that these patients come from different geographical regions and we have not been able to identify any social link. Furthermore they proved to be clearly unrelated after typing with the other 2 techniques.

CONCLUSION

Myriad studies have shown the importance of the molecular typing tool to fully understand routes of transmission. It is also important to be able to have rapid results. Nowadays this is possible thanks to PCR-based techniques. The aim of our preliminary study was to evaluate 2 of them. MIRU-VNTR typing was the most reliable and should be considered the best typing tool for a number of reasons.

MIRU-VNTR has discrimination similar to that of high IS*6110* copy number strains and better for low copy number strains (Cowan *et al.*, 2002; Lee *et al.*, 2002; Valcheva *et al.*, 2008). The typed strains are expressed by a 15 or alternative 24-digit numerical code, corresponding to the number of repeats at each locus. This numerical code is easy to compare and exchange at inter-, and intra-laboratory level. When laboratories have access to an automated sequencer this method is relatively easy to set up and yields results within a day. Alternatively it can be performed with a simple PCR machine and a gel apparatus (Freeman *et al.*, 2006). The storage of data is very easy and data analysis is now further facilitated by the online accessibility of the multifunctional MIRU-VNTR*plus* database kindly supplied by Supply (Allix *et al.*, 2008).

After entering our strains in the database, we carried out a best matches analysis against the 186 reference strains collection representative of the main principal MTBC lineages. A total match was found for a single strain. We then performed the analysis without using the database content, just comparing our strains one against the other to generate a dendrogram describing the genetic relatedness of the 29 isolates under study.

Conversely, only few studies have demonstrated strain-level discrimination for automated rep-PCR with the DiversiLab system (Cangelosi *et al.*, 2004), and one of them proved that rep-PCR does not yield consistent results when it is applied to DNA extracted directly from small amounts of primary diagnostic cultures, requiring well-grown secondary cultures, delaying the time needed to obtain primary results (Ashworth *et al.*, 2008).

The DiversiLab system is a highly integrated, automated and rapid testing platform but it is very expensive, requires training of personnel and did

not prove as valuable as MIRU-VNTR in distinguishing tuberculosis outbreak-related strains from other isolates.

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REFERENCES

ALLAND D., KALKUT G.E., MOSS A.R., MCADAM R.A., HAHN J.A., BOSWORTH W, DRUCKER E., BLOOM, B.R. (1994). Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. *N. Engl. J. Med.* **330**, 1710-1716.

ALLIX C., HARMSEN D., WENIGER T., SUPPLY P., NIEMANN S. (2008). Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolat. *J. Clin. Microbiol*. **46**, 2692-2699.

ASHWORTH M., HORAN K.L., FREEMAN R., OREN E. (2008). Use of PCR-based mycobacterium tuberculosis genotyping to prioritize tuberculosis outbreak control activities. *J. Clin. Microbiol*. **46**, 856-862.

BARNES P.F., CAVE M.D. (2003). Current concepts: molecular epidemiology of tuberculosis. *New England Journal of Medicine*. **349**, 1149-1156.

BRADEN C.R., TEMPLETON G., STEAD W., BATES J.H., CAVE M.D., VALWAY W.E. (1997). Retrospective detection of laboratory cross-contamination of *Mycobacterium tuberculosis* cultures with use of DNA fingerprint analysis. *Clin. Infect. Dis*. **24**, 35-40.

CANGELOSI G.A., FREEMAN R.J., LEWIS K.N., LIVINGSTON-ROSANOFF D., MILAN S.J., GOLDBERG S. (2004). Evaluation of a high throughput repetitive-sequence-based PCR system for DNA fingerprinting of *Mycobacterium tuberculosis* and *Mycobacterium avium complex*. *J. Clin. Microbiol.* **42**, 2685-2693.

COWAN L.S., MOSHER L., DIEM L., MASSEY J.P., CRAWFORD J.T., (2002). Variable number tandem repeat typing of *Mycobacterium tuberculosis* isolates with low copy number of IS6110 by using mycobacterial interspersed repetitive units. *J. Clin.Microbiol*. **40**, 1592-1602.

DEL VECCHIO V.G., PETROZIELLO J.M., GRESS M.J., MCCLESKEY F.K., MELCHER G.P., CROUCH H.K., LUPSKI J.R. (1995). Molecular genotyping of methicillin-resistant Staphylococcus aureus via fluorophore-enhanced repetitive-sequence PCR. *J. Clin. Microbiol.* **33**, 2141-2144.

DYE C., SCHEELE S., DOLIN P., PATHANIA V., RAVAGLIONE M.C. FOR THE WHO GLOBAL SURVEILLANCE AND MONITORING PROJECT. (1999). Global burden of tuberculosis. Estimated incidence, prevalence and mortality by country. *JAMA* **282**, 677-686.

FREEMAN R., KATO-MAEDA M. HAUGE K.A., HORAN K.L., OREN E., NARITA M., WALLIS C.K., CAVE D., NOLAN C. M., SMALL P.M., CANGELOSI G.A. (2005). Use of rapid genomic deletion typing to monitor a tuberculosis outbreak within an urban homeless population. *J. Clin. Microbiol*. **43**, 5550-5554.

HAWKEY P.M., SMITH E.G., EVANS J.T., MONK P., BRYAN G., MOHAMMED H.H., BARDHAN M., PUGH R.N. (2003). Mycobacterial interspersed repetitive unit typingof Mycobacterium tuberculosis compared to IS6110-basedrestriction fragmentlength polymorphism analysis for investigation of apparently clustered cases oftuberculosis. *J. Clin. Microbiol.* **41**, 3514-3520.

HEALY M., HUONG J., BITTNER T., LISING M., FRYE S., RAZA S., SCHROCK R., MANRY J., RENWICK A., NIETO R., WOODS C., VERSALOVIC J., LUPSKI J.R. (2005). Microbial DNA typing by automated repetitive-sequence-based PCR. *J. Clin. Microbiol.* **43**, 199-207.

Kremer K., van Soolingen D., Frothingham R., Haas W.H., Hermans P.W., Martin C., Palittapongarnpim P., Plikaytis B.B., Riley L.W., Yakrus M.A., Musser J.M., van Embden J.D. (1999). Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. *J. Clin. Microbiol.* **37**, 2607-2618

LEE S.G., LYNN L.H., IRENE H.K., RICHARD B., YEWWONG S. (2002). Discrimination of single copy IS6110 DNA fingerprints of Mycobacterium tuberculosis isolates by high resolution minisatellite based typing. *J. Clin. Microbiol*. **40**, 657-659.

MAZARS E., LESJEAN S., BANULS A.L., GILBERT M., VINCENT V., GICQUEL B., TIBAYRENC M., LOCHT C., SUPPLY P. (2001). High-resolution minisatellite-based typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology. *Proc. Natl. Acad. Sci.* USA **98**, 1901-1906.

SECHI L.A., ZANETTI S., DELOGU G., MONTINARO B., SANNA A., FADDA G. (1996). Molecular Epidemiology of Mycobacterium tuberculosis strains isolated from different region of Italy and Pakistan. *J. Clin. Microbiol*. **34**, 1825-1828.

SOLA C., FERDINAND S., SECHI L.A., ZANETTI S., MARTIAL D., MAMMINA C., NASTASI A. FADDA G., NASTOGI N. (2005). Mycobacterium tuberculosis molecular evolution in western Mediterranean Islands of Sicily and Sardinia. *Infection, Genetics and Evolution* **5**, 145-156.

SUPPLY P., ALLIX C., LESJEAN S., CARDOSO-OELEMANN M RUSCH-GERDES S., WILLERY E., SAVINE E., DE HAAS P., VAN DEUTEKOM H., RORING S., BIFANI P., KUREPINA N., KREISWIRTH B., SOLA C., RASTOGI N., VATIN V.,

GUTIERREZ M.C., FAUVILLE M., NIEMANN S., SKUCE R., KREMER K., LOCHT C., VAN SOOLINGEN D. (2006). Proposal for standardization of optimized mycobacterial interspersedrepetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. *J. Clin. Microbiol.* **44**, 4498-4510.

VALCHEVA V., MOKROUSOV I., NARVSKAYA O., RASTOGI N. AND MARKOVA N. (2008). Utility of new 24-locus variable-number tandem-repeat typing for discriminating Mycobacterium tuberculosis clinical isolates collected in Bulgaria. *J. Clin. Microbiol*. **46**, 3005-3011.

VAN EMBDEN J.D., CAVE M.D., CRAWFORD J.T., DALE J.W., EISENACH K.D., GICQUEL B., HERMANS P., MARTIN C., MCADAM R., SHINNICK T.M. (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J. Clin. Microbiol*. **31**, 406-409.

VAN SOOLINGEN D., HERMANS P.W., DE HAAS P.E., SOLL D.R., VAN EMBDEN J.D. (1991). Occurrence and stability of insertion sequences in Mycobacterium tuberculosis complex strains: evaluation of an insertion sequence dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J. Clin. Microbiol*. **29**, 2578-2586.

VERSALOVIC J., KAPUR V., MASON E.O., SHAH U., KOEUTH T., LUPSKI J.R., MUSSER J.M. (1993). Penicillin-resistant Streptococcus pneumoniae strains recovered in Houston: identification and molecular characterization of multiple clones. *J. Infect. Dis*. **167**, 850-856.

VESTAL A.L. (1977). Procedure for isolation and identification of mycobacteria. US Department of Health, Education and Welfare, Publ No CDC 77-8230, Centre for Disease Control (CDC) Atlanta, Georgia.

WORLD HEALTH ORGANIZATION. (2006). Global Tuberculosis Control. WHO Report. World Health Organization, Geneva, Switzerland.