REVIEW ARTICLE

Journal of Pre-Clinical and Clinical Research, 2012, Vol 6, No 1, 1-6
www.jpccr.eu

Genetic methods in molecular epidemiology of tuberculosis

Monika Kozińska, Ewa Augustynowicz-Kopeć

Department of Microbiology, National Research Institute of Tuberculosis and Lung Diseases, Warsaw, Poland

Kozińska M, Augustynowicz-Kopeć E. Genetic methods in molecular epidemiology of tuberculosis. J Pre-Clin Clin Res. 2012; 6(1): 1-6.

Abstract

Introduction: Epidemiological studies of tuberculosis cover a number of medical disciplines, such as clinical medicine, pathology, microbiology, medical statistics, as well as biochemistry and genetics. The rapid development of modern techniques in molecular biology observed in recent years has also allowed their use in the studies of the epidemiology of tuberculosis. These techniques are based on the detection in the mycobacterial genome of characteristic sequences, their organization and polymorphism, and also the identification of genes which determine virulence factors and antibiotic resistance.

Objective: Characteristics of the main genotyping methods applied in molecular epidemiological investigations of tuberculosis.

Brief description of the state of knowledge: Genome sequencing allows the determination of molecular patterns (fingerprinting) of *Mycobacterium tuberculosis* strains analyzed and their discrimination. This is crucial when defining the findings of epidemiological investigations and, above all, recognizing the source of tuberculosis and routes of its transmission. Before molecular methods for epidemiological investigations had been developed, most of the research was limited to discriminating on the basis of biochemical and serological features, phage typing and drug resistance phenotype. With the advent of molecular techniques, epidemiologists began using effective markers to track the transmission and to identify the phylogenetic characteristics of *M. tuberculosis* strains.

Summary: The most important criteria for selection of the method of typing are: the nature of the sample, its size, and the time at which the material was collected. The methods currently used are characterized by varied discriminatory power, the value of which is determined by the degree of clustering/grouping of strains into potential epidemiological groups within which the transmission occurred.

Key words

tuberculosis, molecular epidemiology, genotyping methods

INTRODUCTION

Among many contagious diseases, tuberculosis still remains one of the most frequent causes of morbidity and mortality worldwide. In properly conducted programmes of tuberculosis control it is crucial to use adequate microbiological diagnostics which would enable quick identification of an ill individual, establishment of an optimal therapy in accordance with antibiogram, monitoring of the process of treatment, and facilitation of control over the spread of the disease.

Due to the threat created by a sputum-positive individual, an important task of the medical services is the tracing of his/her contacts with the environment in order to identify all those who might have been exposed to tuberculosis infection.

For many years, knowledge of the epidemiology of tuberculosis has been very limited due to the lack of adequate research methods, and the tracing of the routes of transmission of tubercle bacilli in the human environment was almost impossible. Prior to the implementation of molecular methods, epidemiological investigations were based on the taking of a thorough medical history and comparison of the resistance patterns of the isolated strains of *Mycobacterium tuberculosis*. In the past, due to the low level of laboratory diagnostics, lack of knowledge of the genome of tubercle bacilli, and lack of molecular methods, only hypotheses were posed in epidemiological investigations.

Since the identification in 1882 by Robert Koch of the *Mycobacterium tuberculosis* complex as an etiologic agent of tuberculosis, methods which enable the confirmation of the presence of tubercle bacilli in patient's clinical material have been improved. Highly sensitive methods have been implemented to microbiological diagnostics, simultaneously reducing the time of waiting for the result. The application of genetic methods resulted in tremendous progress. Genotyping allows determination of the molecular patterns (*fingerprints*) of the examined strains of *Mycobacterium tuberculosis* and their differentiation, therefore constituting an objective basis for the identification of strains participating in infection, and excluding strains not related with transmission of the disease.

Studies of tuberculosis transmission in the human environment, and diagnosing cases of an active form of tuberculosis (TB) in patients' surroundings, confirm the high risk of infection among those who remain in close contact with the patient or are family members. Due to the threat of infection resulting from contact with the ill, epidemiological investigations should cover all individuals suspected of tuberculosis, as well as those with its active form confirmed. The tracing of the chain of transmission is aimed at the identification of all people who should be covered with treatment. Such investigations require multi-stage efforts which engage a specialised group of physicians, clinicians, microbiologists and geneticists.

Address for correspondence: Ewa Augustynowicz-Kopeć, Department of Microbiology, National Research Institute of Tuberculosis and Lung Diseases, Warsaw, Poland.
E-mail: e.kopec@igichp.edu.pl

Received: 22 May 2012; accepted: 19 June 2012

These examinations are extremely difficult, require systematic tracing of family contacts, and contacts in the close non-family surroundings, determination of the duration of exposure, estimation of risk and other characteristics [1].

At present, there are very many methods which enable the typing of tuberculosis strains, and there remains only the selection of the typing scheme with the consideration of mainly the type and size of the population examined, and use of the results obtained.

PHENOTYPIC METHODS

Before the development of molecular methods, the majority of epidemiological investigations were based on phage typing. Considering the arduousness of this method and its low sensitivity resulting from the limited number of phages specific for mycobacteria, it was not a suitable epidemiological tool; however, the method was used for the typing of strains forming epidemic foci and investigations of cross-infections in laboratories dealing with the microbiological diagnostics of tuberculosis.

Initially, strains belonging to the *M. tuberculosis* complex were also differentiated based on biochemical characteristics and their different sensitivity to antibiotics. Nevertheless, this was not a reliable method of identification of mycobacterium bacilli due to the limited number of combinations possible. Serologic methods used for typing were useful only in diagnostics, and could not be applied for comparing strains isolated from various patients. Serologic typing allowed only the indication of certain biochemical differences between different strains; however, this was not a suitable tool for their identification [2, 3, 4].

MOLECULAR METHODS

The occurrence of molecular techniques in the epidemiology of tuberculosis provided an opportunity to use effective markers for the tracing of the phenomenon of transmission of the disease in the human environment, and identification of the phylogenetic characteristics of *M. tuberculosis* strains [5]. The presented study describes the most frequently used methods which are most valuable in molecular epidemiological investigations of tuberculosis.

Restriction Fragment Length Polymorpohism (RFLP)

Typing of the *Mycobacterium tuberculosis* strains complex, based on the analysis of nucleic acids, allows the indication of differences within specific sequences, determination of the number of repetitions, and the location of these sequences in the genome. Usually, an initial stage of such an analysis is the digestion of bacilli chromosome with specific restriction enzymes, and subsequent separation of the fragments obtained using agarose gel and the determination of their size. The simplest variant of the RFLP method does not require the stage of hybridization of genome fragments with a specific probe. However, the interpretation of the result is difficult considering the large number of fragments obtained. Then, in electrophoretic separation, complexes are observed consisting of bands of a similar size, and this, in consequence, makes it impossible to detect significant differences between chromosomes [6].

Pulsed Field Gel Electrophoresis (PFGE)

PFGE is a modification of the RFLP method. For restriction analysis, enzymes are used which cut the genome at a limited number of sites, thus obtaining a few fragments of a large size which are then separated under special conditions, i.e. in an alternating electric field. The limitations of this method consist primarily in a relatively low discriminative potential, and the results obtained indicate limited polymorphism between chromosomes of different strains [7].

Methods based on repetitive sequences

The presence of repetitive elements and insertion sequences in the genome of bacilli is commonly considered as a basic criterion for discrimination between strains. In typing the strains of the *Mycobacterium tuberculosis* complex, six repetitive DNA fragments are used (Tab. 1) [8].

Table 1. Repetitive sequences used in typing of mycobacterum bacilli

Repetitive sequence	Scope of occurrence	No. of repeats in genome	Polymorphism level
IS*6110* **(IS*986*, IS*987*)**	*M. tuberculosis*	0-20	High
	M. africanum	0-20	High
	M. bovis	1-20	High
	M. bovis-BCG	1-2	Lack
IS*1081*	*M. tuberculosis*	5-6	Low
	M. africanum	5-6	Low
	M. bovis	5-6	Low
	M. bovis-BCG	5-6	Low
Region DR	*M. tuberculosis*	1	High
	M. africanum	1	High
	M. bovis	1	High
	M. bovis-BCG	1	Lack
MPTR	*M. tuberculosis* complex	±80	Low
PGRS	*M. tuberculosis* complex	26-30	High
MIRU/VNTR	*M .tuberculosis* complex	±40	High

IS*6110* sequence

The IS6110 sequence is most widely used as a probe. This is an insertion sequence which belongs to the IS3 family. According to the species of bacilli, it possesses a proper designation, for *M. tuberculosis* it is described as IS*6110* or IS*986* (the first designation being preferred and commonly applied), while for *M. bovis*-BCG – as IS*987*. Insertion element IS*6110* is 1361bp long and has been identified within the genome of all species of the *M. tuberculosis* complex. The differences between these elements in various strains are limited to several nucleotides only, with a simultaneous variable number of repetitions according to the species and strains of bacilli. The majority of *M. tuberculosis* strains possess in their chromosome 8-15 copies of the IS*6110* sequences, located at various sites of the genome, although strains with one copy are equally often isolated, or sometimes strains devoid of this element [9, 10].

Due to the high polymorphism of the IS*6110* element, methods based on this sequence are characterized by a considerable discriminative potential, and are commonly used in molecular epidemiology.

Despite many advantages, the IS*6110*-RFLP method has certain disadvantages and limitations. The starting material for analysis must be an abundant culture of bacilli, from which large amounts of genomic DNA of high quality and purity must be isolated. The procedure is effort-consuming, and the result obtained after several days. In addition, the

method has a considerable limitation when applied for the typing of strains which, in the genome, possess 0-5 copies of IS*6110* sequences. In such cases, a combination of bands is obtained which are poorly polymorphic in relation to other strains with a low number of IS*6110* copies which, in turn, decreases discriminative potential of the method and decreases its value in typing of such strains. The IS*6110*-RFLP also becomes useless in the case of strains in which the genome is devoid of the IS*6110* element. It is noteworthy that some mycobacteria, other than tubercle bacilli (MOTT), possess in their chromosome many copies of sequences belonging to the IS3 family, which are hybridized with IS*6110* probe, therefore providing a result in the form of bands. Thus, it should be assessed earlier by species identification whether the strain qualifies for IS*6110*-RFLP typing [11, 12].

IS*1081* sequence

The element IS*1081* was described in 1991, and has become an alternative genetic marker for strains possessing only several copies of sequence IS*6110,* or are entirely devoid of these copies. It was found that typing based on IS*1081* has a low discriminative potential resulting from the low transpositional activity of this element. The subsequent limitation was associated with the fact that the method did not allow identification of the species *M. bovis*-BCG from among other species belonging to the *M. tuberculosis* complex [13].

Polymorphic GC-rich repetitive sequences (PGRS)

Very abundant repetitive sequences in the genome *M. tuberculosis* complex are the Polymorphic GC-rich Repetitive Sequences (PGRS). PGRS elements are present at 26-30 sites on chromosome and consist of many tandem repetitions 96 bp in length [14].

Polymorphism of these sequences was used in typing, where GC-rich repetitive sequence contained in the recombinant plasmid pTBN12 is used as the probe. The method is a supplement for analysis of the *M. tuberculosis* complex strains possessing a small number of copies of sequence IS*6110,* or devoid of this sequence.

It is noteworthy that PGRS sequences were also identified in the genome of atypical bacilli [15].

GC-rich sequences were also used in the development of the method IS*6110*-Mtb1-Mtb2 PCR. In two independent PCR reactions, with different starter sets (1. Mtb1+IS1+IS2, 2. Mtb2+IS1+IS2), DNA fragments are amplified located between the IS*6110* sequences and GC-rich regions.

To-date, there are few reports concerning the application of this method for genotyping of the mycobacterium bacilli; nevertheless, it is estimated that this method has a high discriminative potential, comparable to the IS*6110*-RFLP method [16, 17].

Other methods using repetitive elements

Apart from the above-mentioned sequences, other short sequences were identified in the *M. tuberculosis* complex genome, which are used as typing markers. To these sequences belong chromosomal DR (Direct Repeat) regions, positioned in a variable number of repetitions, so-called hot spot regions for integration of the IS*6110* insertion sequences. DR regions consist of short 36 nucleotides DR fragments divided by unique spacers 35-41bp in length. Both the number of copies of DR fragments (maximum 50 repetitions), and the presence of specified spacers are a changeable feature and constitute a basis for indicating differences between strains [18].

Further sequences of a repetitive type are the major polymorphic tandem repeat sequences (MPTR). These are short 10 nucleotides fragments divided by 5 spacers which occur in a variable number of repetitions (maximum 80), both in the genome of the *M. tuberculosis* complex and atypical bacilli. MPTR may be used as an epidemiological marker for the identification of bacilli species; however, their polymorphism is limited [19].

Methods based on amplification reaction

The RFLP method, which has been considered as a golden standard in molecular epidemiology of tuberculosis, requires the obtaining of an abundant culture of bacilli, and this in turn enforces a long time waiting for the result of molecular analysis. For the quickest identification of the pathogen possible, methods are applied based on PCR reaction. The stage of amplification enables typing in situations where culture is not available, but only clinical material from a patient (including also bacterioscopy negative material) [20].

One of these methods is **amplityping**. In this reaction, hybridization starters with the IS*6110* sequence ends are used, and DNA fragments dividing these sequences amplified. However, this method is useless in the case of analyzing a large pool of strains, because of the lack of repeatability of results as a consequence of forming a large amount of non-specific products [21].

The subsequent method indicating polymorphism of chromosome segments between IS*6110* sequences is MPTR. However, due to a limited amount of PCR products obtained, this method has a low discriminative potential and is not suitable for unequivocal determination of affinity between strains.

Among other typing techniques where the IS*6110* sequence is used as a marker, is a group of methods based on ligation of adapters (linkers) to which belong ML PCR (*Mixed-linker PCR*), LM PCR (*Ligation-Mediated PCR*) and Flip PCR (*Fast Ligation-Mediated PCR*). All these methods proceed along a similar scheme and cover the digestion of the genome with restrictive enzyme, ligation of the fragments obtained with adaptor sequences, amplification of ligation products with the use of fluorescence-marked starters complementary to the linker and IS*6110* sequence, and electrophoretic separation of PCR products [22, 23, 24].

Methods based on 16S and 23S rRNA

Spacer regions located between 16S and 23S rRNA coding genes are amplified, and the products obtained are digested with proper restrictases. Considering the limited discriminative potential of the method, its modification was introduced, aimed at duplication of accidental fragments of the entire chromosomal DNA (RAPD*, Random Amplified Polymorphic DNA*). The RAPD analysis provides a high level of discrimination of strains; however, it has a considerable limitation in the form of low repeatability of the results obtained [25].

Spoligotyping

Spoligotyping is one of the most frequently applied methods of typing bacilli of the *Mycobacterium tuberculosis* complex. This method is based on amplification of the chromosomal

DR (*Direct Repeat*) region, consisting of a varied number of short DR sequences (36 bp in length) and unique spacers 35-41 bp in length. The variability of bacilli depends on the number, location, and type of spacers.

Typing by the spoligotyping method does not require complicated procedures and is not time-consuming. Apart from this, it is characterized by high repeatability of results and easiness of their interpretation. Despite many advantages, this method also possesses certain limitations, conditioned primarily by low discriminative potential, assessed as weaker than that of the IS*6110*-RFLP method. Here, a much higher genetic stability of DR region is indicated as the cause, compared to the IS*6110* sequence. Nevertheless, the spoligotyping method is recommended for the typing of strains with a low number of IS*6110* copies (≤ 5) in the genome [26, 27].

Spoligotyping requires a small amount of DNA; therefore, the starting material for analysis may be clinical material from the patient, microscopy smears, as well as fragments of tissues fixed in paraffin blocks. Omission of the stage of waiting for the result of the bacterial culture considerably accelerates the procedure of identification of bacilli, and shortens this procedure from several weeks to 2 days.

The advantage of the spoligotyping method is easiness of recording of the DNA patterns obtained, their cataloguing, and possibility to compare own results with those obtained from other laboratories. The central register of spoligotypes is an international database SpolDB4, describing 1,939 shared types representative of nearly 40,000 strains of *M. tuberculosis* identified in many places worldwide.

At present, the spoligotyping method is applied as a screening test in genotyping tubercule bacillus [28].

Methods based on minisatellite variable number tandem repeat

Analysis of the Variable Number Tandem Repeat (VNTR) is a commonly used method for typing tubercule bacillus. Ministalellite regions consist of many repeated sequences of the length of several dozen base pairs, and the polymorphism of these regions in individual strains of bacilli is a varied number of repetitive fragments.

The VNTR method consists in amplification of the specific locus and, subsequently, an analysis of the size of PCR fragments in agarose gel. The size of the product depends on the number of repetitions of the core motif. The size of the product depends on the number of repetitions of the core motif. The number of repetitions in each loci analyzed provides the ultimate numerical code, the so-called VNTR code [29].

From among the tandem sequences, an analysis is most often based on polymorphism of the motives ETR (Exact Tandem Repeat) and MIRU (Mycobacterial Interspersed Repetitive Units).

Analysis based on ETR sequence polymorphism (most often 5 loci are analyzed: ETR-A, -B, -C, –D, -E) is characterized by low discriminative potential and, therefore, the results require verification based on other genetic markers. For this reason a combination is frequently applied, based on the analysis of both ETR-VNTR and MIRU-VNTR. Among 41 loci which have been identified to-date containing MIRU repeats, 12 hypervariable loci are most often used for genotyping. Nevertheless, some laboratories perform analysis using 15 loci, while others use 24 loci MIRU [20, 30, 31].

MIRU sequences have several dozen base pairs and may occur in a single locus in several or more than 10 repeats. The number of repeats in subsequently analyzed loci is presented in the form of numerical record. MIRU-VNTR typing possesses a high discriminative potential, the results are highly repeatable and easy to interpret. An unequivocal record of the genetic pattern of a strain, similar to spoligotyping, allows the development of the central register of the DNA profiles identified. The discriminative value of the method is evaluated as highly as the value of the IS*6110*-RFLP typing, and both techniques are considered as perfect supplementations of the spoligotyping method [32].

Single Nucleotide Polymorphism SNP

Analysis of single nucleotide polymorphism (SNP) is based of DNA sequencing. Two types of SNP polymorphism have been identified: synonimic (sSNP) and non-synonimic (nsSNP). The difference between them consists in that sSNP does not cause a change in the amino acid, and, therefore, is a neutral modification of the genome. In turn, nsSNP polymorphism leads to an alternation in the amino acid sequence of the protein, and thus may induce a specified phenotypic effect.

Analysis of the polymorphism of several dozen selected loci allows the identification of nucleotide changes in the specified regions of the genome and, in this way, indicates the evolutionary direction in the population of bacilli and their phylogenetic affinity [32, 33, 34].

Importance of genetic methods in molecular investigations in tuberculosis

Methods currently used in molecular investigations are being constantly optimized, contributing considerably to the detection and tracing of the transmission of tuberculosis among humans.

In epidemiological studies of tuberculosis, the IS*6110* RFLP method is considered as the 'golden standard' and is most frequently applied. This method, of a standardized procedure, has a high discriminative capability and high stability of genetic patterns. However, it has some limitations. It requires a large amount (approx. 2 μg) of DNA for analysis, which means the necessity to grow the bacterial culture. The method is complicated from the technical aspect, expensive, and requires modern computer software for the elaboration and comparison of results. In addition, some *M. tuberculosis* complex species show zero or a small number of copies of the IS*6110* sequence, and require discrimination based on alternative methods (Tab.2) [35, 36, 37].

The spoligotyping technique seems to be the most interesting and most frequently used alternative method This method allow the obtaining of patterns (spoligotypes) characteristic of individual strains, and is characterized by a high repeatability of results, mainly due to the stability of the DR region, which is higher than that for the IS*6110* sequence. Spoligotyping, as a method based on PCR requires very small amounts of DNA.

Due to the above, the method may be applied for the detection and identification of the *M. tuberculosis* complex directly in clinical material, omitting the stage of culturing. The results are obtained within 1-2 days. Opposite to the RFLP spoligotyping method, it does not require digestion of chromosomal DNA and conducting of electrophoretic separations; therefore, it may be performed in a shorter

Table 2. Advantages and limitations of selected genotyping methods [35]

Method of typing	Characteristics of method	Limitations of method	Additional comments
IS*6110*-RFLP	Golden standard' in typing *M. tuberculosis* complex strains High variability of DNA patterns obtained for strains with >5 IS*6110* copies Application in molecular epidemiological investigations, tracing of the direction of evolution of strains and phylogenetic affiliations between them	Mycobacterial culture is required for DNA isolation Result after 30-40 days Useless in analysis of strains with ≤5 IS*6110* copies, and devoid of these sequences Lack of a central database of patterns and possibilities to compare results from various laboratories	Detects genetic variability between Beijing strains Transposition and/or deletion of IS*6110* is only one-way and may be the result of evolutionary changes Presence of so-called 'hot spots' of IS6110 sequence integration
Spoligo-typing	One of the simplest methods of mycobacterium tuberculosis genotyping Result in the form of a digital record and cataloguing in an international database; data may be compared between various laboratories Effectiveness of the method is observed with the use of cell lysate and in typing dead cells (microscopic specimens, paraffin blocks) Possesses the character of a screening test	Lower discriminative potential than IS*6110*-RFLP and MIRU-VNTR Makes it impossible to indicate genetic differences between strains of an endemic character	Applied most often for evaluation of geographical distribution of specified strains May indicate false affinity between strains resulting from convergent changes Analysis of spoligotypes is possible based on 2 central databases: SPOTCLUST and SpolDB4
MIRU-VNTR (12 *loci, 15 loci)*	Quick method of high capacity Possesses higher discriminative power of DNA patterns than spoligotyping Digital record of the genetic pattern of a strain Useful in typing a large size population of strains Effectiveness of the method observed with the use of cell lysate Possibility of conducting analysis by 'in-home' or automatic methods	Discriminative potential of the method is close to that of IS*6110*-RFLP (the more loci the higher) Similar DNA profiles may be observed in phylogenetically distant strains Analysis of 12 loci may be insufficient for the indication of differences between endemic strains	Individual loci have their own characteristic 'molecular clock' (genetic variability)
Deligo-typing	In analysis, deletions of chromosome fragments are detected Based on mutiplex PCR, amplification of 43 loci is performed (similar to spoligotyping method) A single deletion enables identification of *M. bovis* BCG Application in studies of phylogenesis and evolution of bacilli, in epidemiological investigations, and in studies of the relationship pathogen-host	Studies are carried out in order to standardize the method	In this method, PCR or DNA microarrays techniques are used Necessity to discriminate unique deletions from repetitive deletions (unique deletions may determine the occurrence of a new phylogenetic branch, while repetitive deletions may evidence a convergent direction of evolution of the strains)
Insertion site typing	Due to the very high discriminative potential, precisely indicates or excludes affinity between strains	Requires preliminary determination of IS*6110* sequence flanking regions	Useful for comparing DNA patterns of individual strains and large size populations
SNP	Method with very high discriminative potential Possibility of automation of the method Applied in phylogenetic studies, in molecular epidemiology, in studies of drug resistance of bacilli, and analysis of the host-pathogen relationship	Analysis covers sequencing of several dozen genomic sites	Polymorphism sSNP does not lead to an alteration in amino acid and, therefore, may be used in studies of genetic affinity of strains Polymorphism nsSNP leads to an alternation in amino acid and this may induce a phenotypic effect, e.g. drug resistance

time and at lower costs. However, many comparisons of the RFLP method and spoligotyping showed that the RFLP method allows a deeper discrimination of the strains of *M. tuberculosis*. Thus, it is postulated that the spoligotyping method is used for the preliminary epidemiological of the collection of strains, while the RFLP method for the discrimination of strains possessing the same spoligotypes. This enables possibly the quickest and cheapest identification of strains belonging to the same epidemiological group. Hybridization patterns obtained by the spoligotyping method are also collected in databases and compared, which allows identification of the types of spoligotypes, and analysis of their spread among the population [38].

In recent years, an increasingly larger number of reports have appeared which indicate that the MIRU-VNTR analysis is the method which effectively discriminates tubercule bacilli. It is based on the identification of minisatellite sequences in the genome of *M. tuberculosis*.

Many analyses confirmed that epidemiological analysis of *M. tuberculosis* strains performed by the MIRU-VNTR method is similarly effective as the RFLP analysis, and in the case of typing strains containing a small number of IS*6110* copies, it is even more useful than the RFLP. In addition, this method is considerably cheaper and less effort consuming, compared to the RFLP, and requires only a small amount of DNA [30, 39].

SUMMARY

Despite great progress in tuberculosis control, improvement in the epidemiological situation in Poland and worldwide, in recent years the problem of tuberculosis has persisted and requires an improvement in the methods for its control, in association with all organizational structures dealing with health protection.

At present, molecular typing of *M. tuberculosis* strains is an inseparable element of epidemiological investigations of tuberculosis. The described techniques of genotyping enable the assessment of variability of the genetic material of strains within the examined population, and determination of the molecular affinity between strains; thus, the possibility of their transmission among abundantly tubercule bacillus positive patients.

Due to this, it is possible to establish the routes of transmission of the disease, its sources in the environment,

and consequently to undertake proper actions in the area of treatment of prophylaxis. At present, the use of molecular methods in the epidemiology of tuberculosis is a necessity in adequately conducted programmes for control of the disease worldwide.

Study financed from grant project No. 404 572740

REFERENCES

1. Van der Zanden AGM, Kremer K, Schouls LM, Caimi K, Cataldi A, Hulleman A, et al. Improvement of differentiation and interpretability of spoligotyping for Mycobacterium tuberculosis complex isolates by introduction of nem spacer oligonucleotides. J Clin Microbiol. 2002; 4: 4628-39.
2. Grange JM, Laszlo A. Serodiagnostic tests for tuberculosis: a need for assessment of their operational predictive accuracy and acceptability. Bull World Health Organ 1990; 68: 571-6.
3. Hoffner SE, Svenson SB, Norberg R, Dias F, Ghebremichael S, Källenius G. Biochemical heterogeneity of Mycobacterium tuberculosis complex isolates in Guinea-Bissau. J Clin Microbiol. 1993; 31: 2215-7.
4. Jones WD. Jr. Bacteriophage typing of Mycobacterium tuberculosis cultures from incidents of suspected laboratory cross-contamination. Tubercle 1988; 69: 43-6.
5. Kanduma E, McHugh TD, Gillespie SH. Molecular methods for Mycobacterium tuberculosis strain typing: a users' guide. J Appl Microbiol. 2003; 94: 781-91.
6. Collins DM, de Lisle GW. DNA restriction endonuclease analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG. J Gen Microbiol. 1984; 130: 1019-21.
7. Varnerot A, Clément F, Gheorghiu M, Vincent-Lévy-Frébault V. Pulsed field gel electrophoresis of representatives of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains. FEMS Microbiol Lett. 1992; 77: 155-60.
8. Dale JW. Mobile genetic elements in mycobacteria. Eur Respir J Suppl. 1995; 20: 633-648.
9. Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for Mycobacterium tuberculosis. J Infect Dis. 1990; 161: 977-81.
10. Hermans PW, van Soolingen D, Bik EM, de Haas PE, Dale JW, van Embden JD. Insertion element IS987 from Mycobacterium bovis BCG is located in a hot-spot integration region for insertion elements in Mycobacterium tuberculosis complex strains. Infect Immun. 1991; 59: 2695-705.
11. Maguire H, Dale JW, McHugh TD, Butcher PD, Gillespie SH, Costetsos A, et al. Molecular epidemiology of tuberculosis in London 1995-7 showing low rate of active transmission. Thorax 2002; 57: 617-22.
12. McHugh TD, Newport LE, Gillespie SH. IS6110 homologs are present in multiple copies in mycobacteria other than tuberculosis-causing mycobacteria. J Clin Microbiol. 1997; 35: 1769-71.
13. van Soolingen D, Hermans PW, de Haas PE, van Embden JD. Insertion element IS1081-associated restriction fragment length polymorphisms in Mycobacterium tuberculosis complex species: a reliable tool for recognizing Mycobacterium bovis BCG. J Clin Microbiol. 1992; 30: 1772-7.
14. De Wit D, Steyn L, Shoemaker S, Sogin M. Direct detection of Mycobacterium tuberculosis in clinical specimens by DNA amplification. J Clin Microbiol. 1990; 28: 2437-41.
15. Ross BC, Raios K, Jackson K, Dwyer B. Molecular cloning of a highly repeated DNA element from Mycobacterium tuberculosis and its use as an epidemiological tool. J Clin Microbiol. 1992; 30: 942-6.
16. Kotłowski R, Shamputa IC, El Aila NA, Sajduda A, Rigouts L, van Deun A, et al. PCR-based genotyping of Mycobacterium tuberculosis with new GC-rich repeated sequences and IS6110 inverted repeats used as primers. J Clin Microbiol. 2004; 42: 372-7.
17. Kozińska M, Augustynowicz-Kopeć E, Zwolska Z, Brzezińska S, Zabost A, Anielak M, et al. Transmission of Mycobacterium tuberculosis among household contacts of patients with tuberculosis. Przegl Epidemiol. 2008; 62: 55-62.
18. van Soolingen D, de Haas PE, Hermans PW, Groenen PM, van Embden JD. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of Mycobacterium tuberculosis. J Clin Microbiol. 1993; 31: 1987-95.
19. Hermans PW, Schuitema AR, Van Soolingen D, Verstynen CP, Bik EM, Thole JE, et al. Specific detection of Mycobacterium tuberculosis complex strains by polymerase chain reaction. J Clin Microbiol. 1990; 28: 1204-13.
20. Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PW, Martín C, et al. 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. 1999; 37: 2607-18.
21. Yuen KY, Chan CM, Chan KS, Yam WC, Ho PL, Chau PY. IS6110 based amplityping assay and RFLP fingerprinting of clinical isolates of Mycobacterium tuberculosis. J Clin Pathol. 1995; 48: 924-8.
22. Graham SM, Gie RP, Schaaf HS, Coulter JB, Espinal MA, Beyers N. Childhood tuberculosis: clinical research needs. Int J Tuberc Lung Dis. 2004; 8: 648-57.
23. Prod'hom G, Guilhot C, Gutierrez MC, Varnerot A, Gicquel B, Vincent V. Rapid discrimination of Mycobacterium tuberculosis complex strains by ligation-mediated PCR fingerprint analysis. J Clin Microbiol. 1997; 35: 3331-4.
24. Reisig F, Kremer K, Amthor B, van Soolingen D, Haas WH. Fast ligation-mediated PCR, a fast and reliable method for IS6110-based typing of Mycobacterium tuberculosis complex. J Clin Microbiol. 2005; 43: 5622-7.
25. Abed Y, Davin-Regli A, Bollet C, De Micco P. Efficient discrimination of Mycobacterium tuberculosis strains by 16S-23S spacer region-based random amplified polymorphic DNA analysis. J Clin Microbiol. 1995; 33: 1418-20.
26. Vitol I, Driscoll J, Kreiswirth B, Kurepina N, Bennett KP. Identifying Mycobacterium tuberculosis complex strain families using spoligotypes. Infect Genet Evol. 2006; 6: 491-504.
27. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J Clin Microbiol. 1997; 35: 907-14.
28. Filliol I, Driscoll JR, van Soolingen D, Kreiswirth BN, Kremer K, Valétudie G, et al. Global distribution of Mycobacterium tuberculosis spoligotypes. Emerg Infect Dis. 2002;8:1347-9.
29. Frothingham R, Meeker-O'Connell WA. Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. Microbiology 1998; 144: 1189-96.
30. Alonso-Rodríguez N, Martínez-Lirola M, Herránz M, Sanchez-Benitez M, Barroso P. Evaluation of the new advanced 15-loci MIRU-VNTR genotyping tool in Mycobacterium tuberculosis molecular epidemiology studies. BMC Microbiol. 2008; 8: 34.
31. Valcheva V, Mokrousov I, Narvskaya O, Rastogi N, Markova N. Utility of New 24-Locus Variable-Number Tandem-Repeat Typing for Discriminating *Mycobacterium tuberculosis* Clinical Isolates Collected in Bulgaria. J Clin Microbiol. 2008; 46: 3005-11.
32. Centers for Disease Control and Prevention (CDC). Trends in tuberculosis – United States, 2007. MMWR Morb Mortal Wkly Rep. 2008; 57: 281-5.
33. Hawkey PM, Smith EG, Evans JT, Monk P, Bryan G, Mohamed HH, et al. Mycobacterial interspersed repetitive unit typing of Mycobacterium tuberculosis compared to IS6110-based restriction fragment length polymorphism analysis for investigation of apparently clustered cases of tuberculosis. J Clin Microbiol. 2003; 41: 3514-20.
34. Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units J Clin Microbiol. 2001; 39: 3563-71.
35. Mathema B, Kurepina NE, Bifani PJ, Kreiswirth BN. Molecular epidemiology of tuberculosis: current insights. Clin Microbiol Rev. 2006; 19: 658-85.
36. Barlow RE, Gascoyne-Binzi DM, Gillespie I. Comparison of variable number tandem repeat and IS*6110*-restriction fragment length polymorphism analyses for discrimination of high- and low-copy-number IS*6110 Mycobacterium tuberculosis* isolates. J Clin Microbiol. 2001; 39: 2453-2457.
37. Cowan LS, Mosher L, Diem L. Variable-number tandem repeat typing of *Mycobacterium tuberculosis* isolates with low copy numbers of IS*6110* by using mycobacterial interspersed repetitive units. J Clin Microbiol. 2002; 40: 1592-1602.
38. Kulkarni S, Sola C, Filliol I, et al. Spoligotyping of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mumbai, India. Res Microbiol. 2005; 156: 588-596.
39. Allix-Beguec C, Harmsen D, Weniger T. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2008; 46: 2692-2699.