ACTA FAC MED NAISS

UDC 579.61:616-078

Review article

ACTA FAC MED NAISS 2009; 26 (4): 225-233

[1]Biljana Miljković-Selimović, [1]Branislava Kocić, [2]Tatjana Babić, [2]Ljiljana Ristić

[1]Department for Microbiology and Immunology, Faculty of Medicine University of Niš, Niš, Serbia
[2]Public Health Institute Niš, Niš, Serbia

BACTERIAL TYPING METHODS

SUMMARY

Bacterial typing methods may be applied in several types of research: in tracking sources and pathways of infection spreading, in examination of infectious diseases, and in population genetics. The method is considered as successful when identified bacterial types are stable and the applied technique has sufficient discriminatory power. The method has to be simple, reproducible, and standardized, too. In bacterial strains typing, both can be used, phenotype and genotype characteristics' analysis. The most applied phenotyping methods are: serotyping, resistotyping, phagotyping, bacteriocin typing, and biotyping. Recently, these methods have been improved by applying molecular biology techniques which allow analyses of the whole cell proteins, outer membrane proteins, cell lipopolysaccharides, and multiple-locus bacterial enzymes. Genotyping methods include determination of plasmid profile, analysis of chromosomal DNA (genome fingerprinting), pulsed-field gel electrophoresis, gene probes, ribotyping, insertional sequences analysis, polymerase chain reaction-based methods, repetitive deoxyribonucleic acid sequences analysis, and deoxyribonucleic acid microarrays. In genotyping methods especially, for profiles comparison of obtained types, the application of specialized software is often necessary. Probably, in the future, genotyping and molecular typing methods will take place instead of classic techniques. Nowadays, using some of these analyses can be expensive and requires specialized staff, but these methods have one unique advantage - high discriminatory level.

Key words: **bacteria, typing methods**

INTRODUCTION

To be successful in resolving outbreaks, microbiologists have to identify epidemically-related isolates among those obtained from different samples (feces, swabs taken from hand, working places, dishes, tools, and food specimens), as there is a possibility to isolate strains of the same bacterial type or serotype which are not connected with the outbreak; that is, to identify a clone of micro-organism related to the outbreak.

According to the clone theory, a term *clone* is used to designate bacterial cultures isolated from different sources, at different locations, and maybe in different period of time, which express so many identical phenotype and genotype characteristics which could be that they are presumed to be derived from a common parent. It seems that the concept of clonal relations among isolated strains was accepted before the term clone itself (1, 2). If isolates, which come from one outbreak, contain identical plasmid profile or plasmid enzyme digestion profile with the same biotype, serotype, and phagotype, they have to be considered as a clone in the strict sense. The

Corresponding author. Biljana Miljković-Selimović • Phone: +38114226448; 0644332238 • Fax:+38184225974 • E-mail: biljams@eunet.rs

former definition describes statistical probability of similarity among the group of independent isolates. Clonality cannot always be totally assured, but it could be applied with an increased probability as a function of discriminatory power of procedures used to describe the examined strain. Since micro-organisms are genetically unstable even without selective pressure in the environment, with mutations, deletions, and ability to obtain new genetic material (e.g. R-plasmid), clonality cannot ever be absolute, thus, it is closely connected with time (2). Cloning can be described as isolation and spreading of an individual biological unit (e.g. bacterial cell) among the population of similar but partially different units (3).

There has been a wide variety of bacterial typing systems currently in use that vary greatly with respect to the effort required, cost, reliability and ability to discriminate between bacterial strains (4). Bacterial typing method is considered successful when the identified bacterial types are stable, the applied technique has sufficient discriminatory power, and the method is simple, reproducible, and standardized. In bacterial strain typing, both phenotyping and genetic procedures can be used.

PHENOTYPING METHODS

The most common phenotyping methods are: serotyping, antibiotic susceptibility testing – resistotyping, phagotyping, bacteriocin-typing, and biotyping. Disadvantage of these commonly applied techniques may be low discriminatory power. In recent years, the quality of microbiological assays has been increasing by using molecular biologic techniques as: whole cell protein profile (WCPP) analysis, outer membrane protein analysis, cell lipopolysaccharide analysis, and electrophoresis of multiple-locus bacterial enzymes.

Analysis of whole cell protein profile, proteins of outer membrane, and cell lipopolysaccharides

The analysis of the whole cell protein profile, proteins of outer membrane, and cell lipopolysaccharides could be presented by SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) profile. SDS PAGE profile is created by destruction of bacteria and separation of soluble strips of extract by molecular weight (5). In SDS PAGE, reproducibility depends on researcher experience and method of bacterial cell lysis. It reaches its maximum when physical sources are applied; it has its minimum when cell is lysed by applying enzymes. Discrimination is a function of differentiation index between isolates. With increasing the number of different bands of two identical isolates, the level of differentiation diminishes. Samples are usually stained by silver. If there are no possibilities to obtain differentiation applying SDS PAGE profile, then immunoblot fingerprinting, which makes that separated extract transferred to nitrocellulose membrane and treated with hyper-immune serum, is used. Then, a typical antigen profile becomes fundamental for typing. The differences in presence, intensity or in position of these antigen bands could be defined as single isolates and compared with control strain. The presence of at least three different antigen patterns is considered as a valid differentiation marker between isolates (6).

Electrophoresis of multiple-locus enzymes

Electrophoresis of multiple-locus enzymes is based on the principle that fine gene mutations of bacterial enzymes (that do not affect the function of enzymes, but influence charge of proteins) could be detected by electrophoresis; such mutations represent an obvious clonal marker. Genes responsible for coding numerous bacterial enzymes are highly polymorphic. Since there is a remarkable differentiation between alleles on structural chromosome genes, the number of clones of one single species is restricted to one hundred up to one thousand (2).

The differentiation among alleles from samples of structural genes enzymes is determined by electrophoresis of multiple-locus enzymes, indexing variations among the sequence of amino acids in enzyme proteins. Analyzing ten to twenty enzymes, the whole bacterial genome can be indexed adequately, and it could be used as a representative measure of genetical relationship among isolates (7). If it is presumed, then the rate of genetical recombination among the species is low, and the method could be used for monitoring bacteria from the same clone. This method could be applied to describe a large number of pathogenic microorganisms: E. coli, Shigella spp., Legionella pneumophila, N. meningitidis, H. influenzae (2).

GENOTYPING METHODS

Genotyping methods include the determination of plasmid profile, analysis of chromosomal DNA (genome fingerprinting), pulsed-field gel electrophoresis (PFGE), gene probes, ribotyping, analysis of insertional sequences (IS), polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified

restriction fragment polymorphism (AFLP), sequence-typing, analysis of repetitive DNA sequences and DNA- microarrays. Techniques could have been classified based on indirect measures of genetic sequence (such as Pulsed-Field Gel Electrophoresis - PFGE) and direct measures of genetic sequence (such as Multilocus Sequence Typing - MLST). For profile comparison of obtained types, application of specialized software is often necessary (8).

Plasmid profile analysis

Since 1970s, with improvements of plasmid isolation methods, examination techniques of total bacterial plasmids contents, as a means of clonality determination, are gaining in importance (1). Plasmid profile analysis is a method of determination the number and size of plasmids in bacterial isolates. It appears that this method offers the easiest way for identification of epidemical strains of intestinal bacteria, pseudomonas, vibriones, staphylococci and streptococci (9, 10). However, even an isolate unlinked with an outbreak could demonstrate the same outbreak-related profile. For that reason, when applying plasmid profile analysis for determining an outbreak strain, the comparison of epidemical and non-epidemical strains isolated in the same period of time is necessary for the evaluation (10). Plasmid profile analysis cannot be successfully applied to S. Enteritidis strains while they usually have one plasmid profile (11).

Chromosomal deoxyribonucleic acid (DNA) analysis

Chromosomal deoxyribonucleic acid (DNA) analysis or genome fingerprinting is based on intact chromosome digestion with restriction endonucleases. Afterwards, the fragments are separated by gel electrophoresis. To evaluate the link between strains, besides the visual typing, the fragment samples in gel could be expressed numerically or processed with computer program which calculates connectivity coefficient between pairs and group of strains. The electrophoretic samples attained in different laboratories or time are difficult to compare due to a large number of restriction fragments. In addition, there is no possibility to measure and record the size of all of them. However, when the restriction fragments are denatured and placed on nitrocellulose paper instead of agarose gel, and then hybridized with DNA probes, attaining of a visually-simple sample is possible. The size of these fragments could be measured and recorded accurately for further comparison (12).

Pulsed-Field Gel Electrophoresis - PFGE

In the PFGE, the entire bacterial genome is cut with one or two restriction enzymes. The length of the pieces of DNA resulting from this digestion will provide a pattern that differentiates between two bacterial strains. If cells originate from identical bacterial isolates, their chromosomes will have the same nucleotide sequence. Since the DNA segments are released by enzymes that recognize very large segments of DNA, a special form of electrophoresis is needed to separate them. Led by an electric current, the negatively charged DNA is forced to travel through the gel in the direction of the positive pole. Smaller pieces of DNA will move more freely than larger pieces, and therefore will travel further in the direction of the current. To aid the passage of large DNA fragments in gel, the used current is periodically reversed in polarity compelling the fragments of DNA to move in the predominant direction towards the bottom of the gel. Based on this principle, PFGE is able to determine the lengths of the DNA fragments in relation to the other samples used. Also, it can give an estimate of the lengths of the pieces by comparing their position in the gel relative to a 'ladder', a mix of fragments of known lengths (13). PFGE can be added to the list of Gram-positive organisms as Enterococcus spp., Clostridium spp., Staphylococcus spp., Streptococcus spp., as well as Gram-negative organisms: Acinetobacter spp., Bacteroides spp., Bordetella pertussis, Borrelia burgdorferi Burkholderia cepacia, Campylobacter spp., Escherichia coli, Mycobacterium spp., Neisseria spp., Salmonella spp., Shigella spp., Vibrio cholerae, Stenotrophomonas maltophilia, Yersinia pestis, etc. (14).

DNA probes in detection and typing of microorganisms

In order to discover an unknown microorganism, examining of DNA is based on the affinity of one DNA chain to bind to complementary chain which forms the hybrid made of double stranded DNA. In this case, a part of single nucleotide chain which originates from a DNA of certain microorganism, known as probe, is used for discovering other microorganisms which contain the same gene. Theoretically, any part of DNA which is characteristic of a certain group of microorganisms could be used as a probe. Specific and universal probe could be used in molecular genetic identification and typing. Universal probes are: ribosomal RNA (16 and 23S) from some species of E. coli, universal and accidentally cloned DNA fragments which could be used in typing of restriction DNA samples (15). Though, specific

DNA hybridization is fundamental as a diagnostic tool. Specific probes are attained from cloned DNA fragments of the known genes or synthetic oligonucleotides. In this case, the probe will "detect" and "identify" a bacterium even in a mixed culture. For identification of S. enterica subsp. enterica serotype Enteritidis (S. Enteritidis), one specific, synthetic, oligonucleotide probe (SE – probe) completely homologous to SpvA gene of S. Enteritidis is described (16). Cryptic plasmid found in many N. gonorrhoeae strains, it specifically hybridizes just with gonococci, but not with other neisseria species or other bacteria, and could detect N. gonorrhoeae in urethral secretion. This specificity is used for development of DNA probes which distinguish enterotoxigenic E. coli from non-pathogenic strains. In this case, DNA probe consists of cloned genetic sequences which code the formation of three enterotoxin types: thermolabile (LT), thermostabile I and II (STI and STII), and bacteria which contain these genes will react with the probe consequently. Due to enormous similarity among thermolabile enterotoxins produced by E. coli and Vibrio cholerae, the probe for LT E. coli could be applied for detection of V. cholerae, too. The examples show the use of cloned genes both for detection pathogenic strains and determination of a strain ability to create toxins (9).

Ribotyping

Ribotyping is a method which, as a universal hybridization DNA probe for rRNA of any bacterial strain, uses rRNA (16 and 23S) of E. coli. After cutting bacterial DNA by endonucleases, the transfer of DNA fragments is made through membranes as well as hybridization with rRNA of E. coli. While some genera are homogenic where all the strains of species have unique restriction rRNA gene profile, the other species show genetic heterogeneity characterized by thermal instability of hybridized DNA. Different strains of such species could give different restriction samples of rRNA genes. In that case, the method is more considered as typing than identifying (16). Ribotyping that is determining of restriction fragment length polymorphism of rRNA genes could be also applied for classification of several Salmonella serotypes (17).

Insertional Sequences - IS

Insertional sequences (ISs), whose lengths vary from 800 to 1,400 bp, could be integrated at various sites on bacterial or phage genomes and could cause polar mutations phenomenon. It is known that these are structurally defined nucleotide sequences which repeat in opposite direction along the plasmid genome. They have the ability not only to disable a host gene function but to affect a function of genes distal to the insertion point regarding to operon promoter. Presence of ISs in bacterial genome is used for DNA probes creating. For S. Enteritidis, it is probe IS200 (18).

Polymerase Chain Reaction – PCR

PCR is defined as a method for the in vitro production of large quantities of DNA specific fragment with a given length and sequence from a very small sample. It is possible due to Taq polymerase enzyme isolated from Thermus aquaticus, bacterium which synthesizes DNA chains. In contrast to the other, this polymerase is characterized by its high temperature stability (up to 97ºC). The process of DNA sequence synthesis has three stages: DNA heat denaturation at 92 – 96ºC; binding of complementary oligonucleotides (deoxynucleotide triphosphates) - primers which determine the beginning and the end of the copying site of DNA; DNA synthesis catalyzed by Taq polymerase. One primer binds to the 3' end of a given DNA sequence, and another to the 5' end at second chain of a given DNA sequence. The reaction mixture is warmed up at various cycle stage-specific temperatures.

PCR is used due to its advantages as: specificity, rapid DNA amplification, capability to amplify only desired DNA sequence. Disadvantages of PCR method are: the cost of reagents and necessary equipment, and high sensitivity of PCR reactions which can lead to false-positive results caused by contamination. It can be used in diagnostic and research purposes for detection and identification of all agents which contain DNA: plants, animals and humans. PCR method is used for determination gene sequences whose proteins are characterized by partially known amino acid sequence, for mutation analysis and definition of sequences located near incorporated virus or gene (19).

A rapid PCR assay that types strains of Mycobacterium tuberculosis by generating distinct DNA fingerprints directly from primary cultures has been developed. The assay can be converted to a computer-automated system so that unknown-specimen fingerprints can be identified by computer comparison to a database of M. tuberculosis strain fingerprints (20).

Real – time PCR

The principle of this technique is the same as for classical PCR method, but in real-time PCR, the synthesized products from every cycle are detected

because they are marked with fluorescent dye. This method enables the determination of relative sample quantities by their comparison in contrast to traditional quantitative PCR which determines the final sample quantity. Real time-PCR with fluorescens energy transfer (FRET) assay and a melting curve analysis of the amplification products allows specific detection of Helicobacter pylori as well as its susceptibility to clarithromycin. Using specific primers, it is possible to detect point mutation in which the resistance to clarithromycin is confirmed, or mutation in H. pylori responsible for quinolones resistance (21).

Reverse Transcription – PCR (RT – PCR)

RT – PCR is a method for theoretical evidencing of one single iRNA molecule. The reaction has two stages. In the first stage RNA is transcribed to cDNA (cDNA: DNA complementary to RNA), by reverse transcriptase enzyme responsible for synthesis of DNA from an RNA template in the presence of deoxynucleotide triphosphate. In this way, a product complemented to initial RNA is created. In the next stage, it comes to amplification of transcribed cDNA, and the analysis of obtained PCR products is performed. Possibility to work on very small sample sizes is the advantage of RT-PCR method (19). This method is applied in detection and typing of extended-spectrum beta-lactamases strains of Shigella sonnei (22).

Multiplex PCR

In multiplex PCR method, simultaneous binding of primers to several genes (two – three) is applied, which leads to amplification and detection of several genes at the same time. This method is used in identification of both thermophilic campylobacters as well as other diarrheagenic bacteria (23). The multiplex PCR in characterization of methicillin-resistant Staphylococcus aureus (MRSA) was shown to be rapid, solid, and capable in a single assay of identifying five structural types of the mec, drug resistance determinant, which has already been seen among MRSA characterized earlier (24).

Restriction Fragment Length Polymorphism – RFLP

The method Restriction Fragment Length Polymorphism (RFLP) detects a difference in homologous DNA sequences by the presence of fragments of different lengths after digestion of the DNA samples with specific restriction endonucleases. The resulting DNA fragments are then separated by length through agarose gel electrophoresis, and transferred to a membrane via the Southern blot. Hybridization of the membrane to a labeled DNA probe determines the length of the fragments which are complementary to the probe. A RFLP occurs when the length of a detected fragment varies between individuals. Besides determination of the differences among microorganism strains, the method could be used in forensic medicine to determine heredity between people (25). The PCR-RFLP method in Mycobacterium tuberculosis investigation is considered to be rapid, with results within 1 to 2 days of the request being received and reproducible. The restriction enzyme analysis stage has improved the efficiency of the technique (26). PCR-RFLP analysis of sterile body fluids may be a useful method for the diagnosis of mycobacterial infections and for differentiation of mycobacterial species other than M. tuberculosis (27).

Random Amplified Polymorphic DNA - RAPD

Testing of the polymorphisms of Random Amplified Polymorphic DNA is called RAPD. That assay is based on the amplification of random genomic DNA segments with single primers of arbitrary nucleotide sequence. Technique employs the polymerase chain reaction (PCR) to amplify DNA fragments. Instead of the usual pair of primers directed at a specific target, a single randomly selected primer is used which binds at multiple sites along the genome at the low annealing temperatures used; products are produced between primers binding in close proximity to opposite DNA strands. Primers detect polymorphisms in the absence of specific nucleotide sequence information, and the polymorphisms among the amplification products are detected frequently, are useful as genetic markers, and can be detected through examination of an ethidium bromide-stained agarose gel. It can be used to construct genetic maps in a variety of species (28). Compared with RFLP, RAPD is more rapid and less technically demanding to perform. RAPD has been used successfully to type a variety of different bacteria e. g., Proteus mirabilis (29, 30).

Amplified restriction fragment polymorphism - AFLP

Amplified restriction fragment polymorphism (AFLP) is a PCR-based DNA fingerprinting technique. In AFLP analysis, bacterial genomic DNA is digested with restriction enzymes, ligated to adapters, and a subset of DNA fragments are amplified using primers containing adapter defined sequences which do not require prior knowledge of

nucleotide sequence. Polymorphisms are detected by differences in the length of the amplified fragments in PAGE and are assessed as the presence or absence of bands (31,32). Polymorphisms of different E.coli strains or Agrobacterium tumefaciens strains were demonstrated as distinct, unique bands in a denaturing sequencing gel using AFLP (33).

Analysis of repetitive DNA sequences

Repetitive DNA sequences are found in many bacterial species. They are often incorrectly copied, which results in shortening or lengthening of the repeat region due to deletion or insertion of repeat units. Tandem repeats that represent a single locus are called variable number of tandem repeats (VNTR) locus. This method relies on PCR amplification of multiple loci using primers specific for the flanking regions of each repeat locus and on the determination of the sizes of the amplicons, which reflect the numbers of the targeted VNTR copies. VNTR typing is technically flexible, as sizing can be done using capillary or gel electrophoresis or nondenaturing high-performance liquid chromatography. The results can be expressed as numerical codes and are therefore very easy to compare and exchange. The number of repeat units can be highly variable, making this method highly discriminatory (34). Combined analysis of more VNTR areas is termed as multilocus variable number tandem repeat analysis, MLVA. MLVA has been successfully developed for Salmonella Typhimurium (35).

Sequence-typing methods

In this method, protocols are usually based on PCR amplification of the target gene(s), followed by restriction fragment length polymorphism (RFLP) analysis of the PCR products obtained, using recommended restriction enzymes. Single-locus sequence typing can be applied to specific gene as emm typing of Streptococcus pyogenes (36), spa typing of Staphylococcus (37), and fla typing of Campylobacter (38). Multi-locus sequence typing (MLST) can be applied to housekeeping genes: about seven to eight house-keeping genes are commonly used in the laboratories. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). MLST requires only the ability to amplify DNA fragments by PCR and to sequence the fragments, using an automated sequencer, or manually (39). For each of the housekeeping gene, the different sequences are assigned as alleles and the alleles at the loci provide an allelic profile. The identification marker for strain typing can be a series of profiles. Sequences that differ at even a single nucleotide are assigned as different alleles. The large number of alleles at each of the loci provides the ability to distinguish a large number of different allelic profiles, and a strain with the most common allele at each locus would only be expected to occur at very low chance. The relatedness of isolates is displayed as a dendrogram constructed using the matrix of pair-wise differences between their allelic profiles. MLST can be applied to almost all bacterial species and other haploid organisms, including those that are difficult to cultivate. The important advantage of MLST over other molecular typing methods is that sequence data are portable between laboratories (39). The accumulation of nucleotide changes in housekeeping genes is a relatively slow process and the allelic profile of a bacterial isolate is sufficiently stable over time for the method to be ideal for global epidemiology. MLST was originally developed for procaryotic bacteria as Campylobacter jejuni and H. pylori, but has recently also been used for characterization of several eukaryotic species including Candida albicans and Batrachochytrium dendrobatidis (40). It is considered that MLST will place strains into fewer, larger groups than typing techniques based on more variable genes, such as PFGE, which means that PFGE is generally more discriminatory than MLST (4).

DNA Microarrays

Gene expression profile analysis technique is called DNA microarray or DNA chip. This method allows rapid and real analysis of a large number of different mRNA. It is necessary to isolate mRNA from a given sample which is followed by complementary DNA (cDNA) synthesis. After the beginning of cDNA synthesis, its first chain has been marked and denatured to a single stranded DNA. It presents the origin of targeted sequences. In the next step, the marked cDNA is hybridized with nucleotides of known structure obtained commercially. They are placed in small tubes where the reaction takes a place. If the gene segment is recognized, it is followed by the process of hybridization which is recorded and compared with other samples. Relative probe hybridization signals can be measured when two or more different preparations are labeled with distinguished fluorophores. In this way, based on the hybrid, which reveals the presence of mRNA, information about gene expression is generated (19). Microarrays comprised of several thousand probes on glass slides can be manufactured in the laboratory using robotic arrayers. According to Van Ijperen and Saunders,

(2004) microarrays that include probes for every gene within a genome provide excellent comparative data, although a focus on variable genes may be more useful for typing purposes (41). An allele-specific oligonucleotide microarray was developed for rapid typing of pathogens based on analysis of genomic variations. In the signature concept, all strains are interrogated by hybridizing their amplified DNA to a multiple probe sequences. Allele-specific oligonucleotide probe sequences targeting each of these variable regions were synthesized and included in a custom fiber-optic array. For each locus, a set of specific probe sequences is selected. Such hybridization gives a binary signal/no signal response to each of the probes. Using this strategy for multiple loci, many pathogens or microorganisms could be classified using a limited number of probes (42).

Comparison of common bacterial typing techniques can be performed according to relative discriminatory power, reproducibility, repeatability, and whether they give information on dispersed or focal parts of the genome, time required and cost. For example, sequencing of the entire genome of bacteria could detect very small differences between strains as changes in gene sequence which do not cause changes in the expressed proteins. However, the sequencing of entire genome, although with high discriminatory power and repeatability, required months to years with very high relative cost. In 2005, total genomic sequencing cost roughly ~$100,000 to $500,000 per strain (4). It is thought that sequence-based methods are most repeatable and reproducible while gel-based methods are less so, because of the inherent variability of the technique (43).

Understanding the strengths and weaknesses of the chosen bacterial typing technique enhances interpretation and generalization of study results (4). Even though it is difficult to recommend a technique that match bacterium, this brief paper reviews current methods and available techniques with examples given. Likely, in the future, molecular typing methods could replace resistotyping, serotyping, phagotyping, bacteriocin typing, and the other classic ones. At the moment, using some of them is too expensive and requires specialized staff, but these methods have one unique advantage: a high level of discrimination.

REFERENCES

1. Ørskov F, Ørskov I. Summary of a workshop on the clone concept in the epidemiology, taxonomy, and evolution of the *Enterobacteriaceae* and other bacteria. J Infect Dis 1983; 148: 346-57.

2. Eisenstein BI. New molecular techniques for microbial epidemiology and the diagnosis of infectious diseases. J Infect Dis 1990; 161: 595-602.

3. Eisenstein BI, Engleberg CN. Applied molecular genetics: new tools for microbiologists and clinicians. J Infect Dis 1986; 153: 416-29.

4. Foxman B, Zhang L, Koopman JS, Manning SD, Marrs C. Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiol Perspect Innov 2005; 2: 10. Published online 2005 November 25. doi: 10.1186/1742-5573-2-10.

5. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 1981; 112(2): 195–203.

6. Burnie JP, Lee W. A comparison of DNA and immunoblot fingerprinting of the SII biotype of coagulase negative staphylococci. Epidemiol Infect 1988; 101(2): 203–212.

7. Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol 1986; 51: 873-84.

8. Jolley KA, Maiden MCJ. AgdbNet – antigen sequence database software for bacterial typing. BMC Bioinformatics 2006; 7: 314. doi:10.1186/1471-2105-7-314.

9. Tompkins L. DNA methods in clinical microbiology. in: Lenette EH (ed), Manual of clinical microbiology (4th edition), American Society for Microbiology, Washington D.C., 1985: 1023-28.

10. Wachsmuth K. Molecular epidemiology of bacterial infections: examples of methodology and of investigations of outbreaks. Rev Infect Dis 1986; 8: 682-92.

11. Miljković-Selimović B, Lepšanović Z, Babić T, Kocić B, Ranđelović G. [Plasmid profile analysis in identification of epidemic strains of *Salmonella enterica* serovar Enteritidis]. Vojnosanit pregl 2008; 4: 303-7. (in Serbian)

12. Li W, Raoult D, Fournier PE. Bacterial strain typing in the genomic era. FEMS Microbiol Rev 2009. (in press)

13. Schwartz DC, Saffran W, Welsh J, Haas R, Goldenberg M, Cantor CR. New techniques for purifying large DNAs and studying their properties and packaging. Cold Spring Harb Symp Quant Biol 1983; 47 Pt 1:189-95.

14. Tenover CF, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting Chromosomal DNA Restriction Patterns Produced by Pulsed-Field Gel Electrophoresis: Criteria for Bacterial Strain Typing. J Clin Microbiol 1995; 33(9): 2233–9.

15. Altwegg M, Mayer LW. Bacterial molecular epidemiology based on a nonradioactive probe complementary to ribosomal RNA. Res Microbiol 1989; 140(4-5): 325–333.

16. Hanes DE, Koch WH, Miliotis MD, Lampel KA. DNA probe for detecting *Salmonella enteritidis* in food. Mol Cell Probes 1995; 9: 9-18.

17. Usera MA, Popovic T, Bopp CA, Strockbine NA. Molecular subtyping of *Salmonella enteritidis* phage type 8 strains from the United States. J Clin Microbiol 1994; 32: 194-8.

18. Millemann Y, Lesage MC, Chaslus-Dancla E, Lafont JP. Value of plasmid profiling, ribotyping, and detection of IS200 for tracing avian isolates of *Salmonella typhimurium* and *S. enteritidis*. J Clin Microbiol 1995; 33: 173-9.

19. Janeway C, Travers P, Walport M, Shlomchik M, editors. Immunobiology: The Immune System in Health and Disease (5th ed). New York, NY: Garland Publishing/Taylor & Francis Group, Churchill Livingstone; 2001.

20. Neimark H, Ali Baig M, Carleton S. Direct Identification and Typing of *Mycobacterium tuberculosis* by PCR. J Clin Microbiol 1996; 34 (10): 2454–9.

21. Glocker E, Kist M. Rapid detection of point mutation in thr *gyr*A gene of *Helicobacter pylori* conferring resistance to ciprofloxacin by a fluorescence resonance energy transfer-based real-time PCR approach. J Clin Microbiol 2004; 42: 2241-6.

22. Gómez-Duarte OG, Bai J, Newell E. Detection of *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagn Microbiol Infect Dis 2009; 63(1): 1-9.

23. Sabra AH, Araj GF, Kattar MM, Abi-Rached RY, Khairallah MT, Klena JD, Matar GM Molecular characterization of ESBL-producing Shigella sonnei isolates from patients with bacilliary dysentery in Lebanon. J Infect Dev Ctries 2009; 3(4):300-5.

24. Oliveira DC, Lencastre H. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2002; 46 (7): 2155-61.

25. Tanksley SD, Young ND, Paterson AH, Bonierbale MW. RFLP mapping in plant breeding: new tools for an old science. Biotechnology 1989; 7:257–264.

26. Yates MD, Drobniewski FA, Wilson SM. Evaluation of a Rapid PCR-Based Epidemiological Typing Method for Routine Studies of *Mycobacterium tuberculosis* J Clin Microbiol 2002; 40(2): 712–4.

27. Cho CH, Han SH, Chin BS, Choi SH, Lee HS, Kim CO, Kim MS, Choi JY, Song YG, Kim JM. Diagnosis and species identification of mycobacterial infections by polymerase chain reaction-restriction fragment length polymorphism analysis of sterile body fluids. Korean J Intern Med 2009; 24(2):135-8.

28. Williams' JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 1990; 18 (22): 6531-5.

29. Cancilla MR, Powell IB, Hillier AJ, Davidson BE. Rapid genomic fingerprinting of *Lactococcus lactis* strains by arbitrarily primed polymerase chain reaction with P3' and fluorescent labels. Appl Environ Microbiol 1992; 58: 1773-5.

30. Michelim L, Muller G, Zacaria J, Delamare AP, Costa SO, Echeverrigaray S. Comparison of PCR-based molecular markers for the characterization of Proteus mirabilis clinical isolates. Braz J Infect Dis 2008; 12(5):423-9.

31. Vos P, Hogers R, Bleeker M, Rijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 1995; 23:4407–4414.

32. Meudt HM, Clarke AC. Almost forgotten or latest practice? AFLP applications, analyses and advances. Trends Plant Sci 2007; 12(3):106-17.

33. Lin J-J, Kuo J, Ma J. A PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria. Nucleic Acids Res 1996; 18 : 3649-3650.

34. Ramazanzadeh R, McNerney R. Variable Number of Tandem Repeats (VNTR) and its application in bacterial epidemiology. Pak J Biol Sci 2007; 10(16): 2612-21.

35. Torpdahl M, Sørenson G, Linstedt BA, Nielsen EM. Tandem repeat analysis for surveillance of human *Salmonella typhimurium* infections. EID 2007; 3: 388-395.

36. Yoonim N, Olive C, Pruksachatkunakorn C, Good MF, Pruksakorn S. M protein typing of Thai group A streptococcal isolates by PCR-Restriction fragment length polymorphism analysis. BMC Microbiol 2005; 5: 63.

37. Mehndiratta PL, Bhalla P, Ahmed A, Sharma YD. Molecular typing of methicillin-resistant *Staphylococcus aureus* strains by PCR-RFLP of SPA gene: a reference laboratory perspective. Indian J Med Microbiol 2009; 27(2): 116-22.

38. Djordjevic SP, Unicomb LE, Adamson PJ, Mickan L, Rios R; Australian Campylobacter Subtyping Study Group.Clonal complexes of *Campylobacter jejuni* identified by multilocus sequence typing are reliably predicted by restriction fragment length polymorphism analyses of the flaA gene. J Clin Microbiol 2007; 45(1):102-8.

39. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A 1998; 95(6): 3140-5.

40. Urwin R, Maiden MCJ. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol 2003; 11(10): 479-487.

41. Van Ijperen C, Saunders NA. Microarrays for bacterial typing: realistic hope or holy grail? Methods Mol Biol 2004; 266: 213-27.

42. Shepard JRE, Danin-Poleg Y, Kashi Y, Walt DR. Array-Based Binary Analysis for Bacterial Typing. Anal Chem 2005; 77 (1): 319 -326.

43. van Belkum A. High-throughput epidemiologic typing in clinical microbiology. *Clin Microbiol Infect* 2003; 9:86–100.

METODE TIPIZACIJE BAKTERIJA

[1]Biljana Miljković-Selimović, [1]Branislava Kocić, [2]Tatjana Babić, [2]Ljiljana Ristić

[1]Institut za mikrobiologiju i imunologiju, Medicinski fakultet u Nišu, Niš, Srbija
[2] Institut za javno zdravlje Niš, Niš, Serbia

SAŽETAK

Tipizacija bakterija se primenjuje u mnogim ispitivanjima, počev od praćenja izvora i puteva prenošenja uzročnika, preko ispitivanja infektivnih bolesti do populacione genetike. Da bi metoda tipizacije bila uspešna, identifikovani tipovi bakterija bi trebalo da budu stabilni, tehnika bi trebalo da ima dovoljnu moć diskriminacije, da bude jednostavna i reproducibilna, a metod da je standardizovan velikim brojem ponovljenih testiranja. U tipizaciji bakterijskih sojeva mogu da se koriste metode tipizacije fenotipskih i genotipskih osobina. Metode fenotipizacije koje se najčešće primenjuju su: serotipizacija, rezistotipizacija, fagotipizacija, bakteriocin-tipizacija i biotipizacija. Poslednjih godina se ove metode poboljšavaju primenom tehnika molekularne biologije, što omogućava ispitivanje ukupnih ćelijskih proteina, proteina spoljne membrane, ćelijskih lipopolisaharida, analizu multilokusnih bakterijskih enzima. Genotipske metode tipizacije obuhvataju: određivanje plazmidskog profila, analizu hromozomske dezoksiribonukleinske kiseline (fingerprinting genoma), elektroforezu u pulsirajućem polju električne struje, genske probe, ribotipizaciju, insercione sekvence, metode bazirane na lančanoj reakciji polimeraze, analizu repetitivnih sekvenci dezoksiribonukleinske kiseline i „microarrays". Za upoređivanje profila dobijenih tipova često je neophodno korišćenje specijalizovanih softvera. Verovatno će jednog dana metode genotipizacije i molekularne tipizacije zameniti klasične metode. Možda je sada korišćenje neke od ovih metoda skuplje i zahteva visoko specijalizovano osoblje, ali ove metode imaju jedinstvenu prednost: visok stepen diskriminacije.

Ključne reči: **bakterije, metode tipizacije**