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Review

Principles and applications of Ligation Mediated PCR methods for DNA-based typing of microbial organisms*

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A significant number of DNA-based techniques has been introduced into the field of microorganisms' characterization and taxonomy. These genomic fingerprinting methods were developed to detect DNA sequence polymorphisms by using general principles, such as restriction endonuclease analysis, molecular hybridization, and PCR amplification. In recent years, some alternative techniques based on ligation of oligonucleotide adapters before DNA amplification by PCR, known as Ligation-Mediated PCR methods (LM PCR), have been successfully applied for the typing of microorganisms below the species level. These molecular methods include: Amplified Fragment Length Polymorphism (AFLP), Amplification of DNA fragments Surrounding Rare Restriction Sites (ADSRRS), PCR Melting Profiles (PCR MP), Ligation Mediated PCR/Shifter (LM PCR/Shifter), Infrequent-Restriction-Site Amplification (IRS PCR), double digestion Ligation Mediated Suppression PCR (ddLMS PCR). These techniques are now applied more and more often because they involve less time, are comparably inexpensive, and require only standard lab equipment. Here, we present a general review of this group of methods showing their possibilities and limitations. We also identify questions and propose solutions which may be helpful in choosing a particular LM PCR method for the achievement of the required goal.

Key words: rapid methods, molecular epidemiology, genotyping, microbial phylogenetics, PCR (polymerase chain reaction)

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INTRODUCTION

Genomic typing focuses on the analysis of DNA of microorganisms at the level of a strain or subspecies, and detects many classes of genomic variations, mainly intragenomic recombination and horizontal gene transfer events.

Currently, genetic typing of microorganisms is widely used in several major fields of microbiological research, as a tool in monitoring of hospital infections, for epidemiological investigations to confirm or negate relationships of clonal strains, in the study of pathogenesis, phylogenetic studies, population genetics of microorganisms or taxonomy. DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. A variety of DNA techniques is currently available, most of which use PCR for detection of fragments.

The choice of a fingerprinting technique depends on the type of application, such as intraspecific diversity analysis (strain typing to study genetic relationships and bacterial population dynamics, outbreak management for identification of the track and source of bacterial infection) or identification at the species level. Ideally, a fingerprinting technique should require no prior investments in terms of sequence analysis. However, knowledge of the species' variability, CG-content percentage or specific restriction enzyme profile is helpful.

A number of fingerprinting methods which meet these requirements has been developed. Fingerprints are obtained by visualizing only a very minor part of the genome, although with an appropriate level of discriminatory power. In addition to this, they should be relatively rapid and cheap, especially in large-scale population genetic studies. In this view, a particular interest can be observed in rapidly developing techniques which employ oligonucleotide adapters and are known as Ligation-Mediated PCR methods (LM PCR). They are known as a high versatile group of techniques for genomic typing and their versatility arises from the fact that they do not require prior knowledge of the sequence and a virtually unlimited number of restriction fragments can be detected and analyzed in genomes.

FRAMEWORK OF LM PCR METHODS — A GENERAL STRATEGY

LM-PCR is comprised of a group of genome fingerprinting techniques based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion. The fingerprint pattern does not include any specific information about the species or genus of the tested strain; therefore they require the use of the purified DNA from correctly identified species of bacteria, yeast or other microorganisms (thus also the need for pure microorganism culture selection). For all LM PCR methods it is necessary to use intact, non-de-

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Abbreviations: ADSRRS, Amplification of DNA fragments Surrounding Rare Restriction Sites; AFLP, Amplified Fragment Length Polymorphism; ddLMS PCR, double digestion Ligation Mediated Suppression PCR, *rec*A-ddLMS PCR, double digestion Ligation Mediated Suppression PCR for *rec*A gene; *rrn*-ddLMS PCR, double digestion Ligation Mediated Suppression PCR for *rrn*-locus; FLAP, Fast Ligation Amplification Polymorphism; FLiP, Fast Ligation-Mediated PCR; IRS PCR, Infrequent-Restriction-Site Amplification; LM PCR, Ligation-Mediated PCR; LM PCR/Shifter, Ligation Mediated PCR/Shifter; MIRU–VNTR, Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeat; PCR, Polymerase Chain Reaction; PCR MP, PCR Melting Profiles; PFGE, Pulse Field Gel Electrophoresis; REA PFGE, Restriction Enzyme Analysis with Pulse Field Gel Electrophoresis; RFLP, Restriction Fragment Length Polymorphism; T_d, denaturation temperature

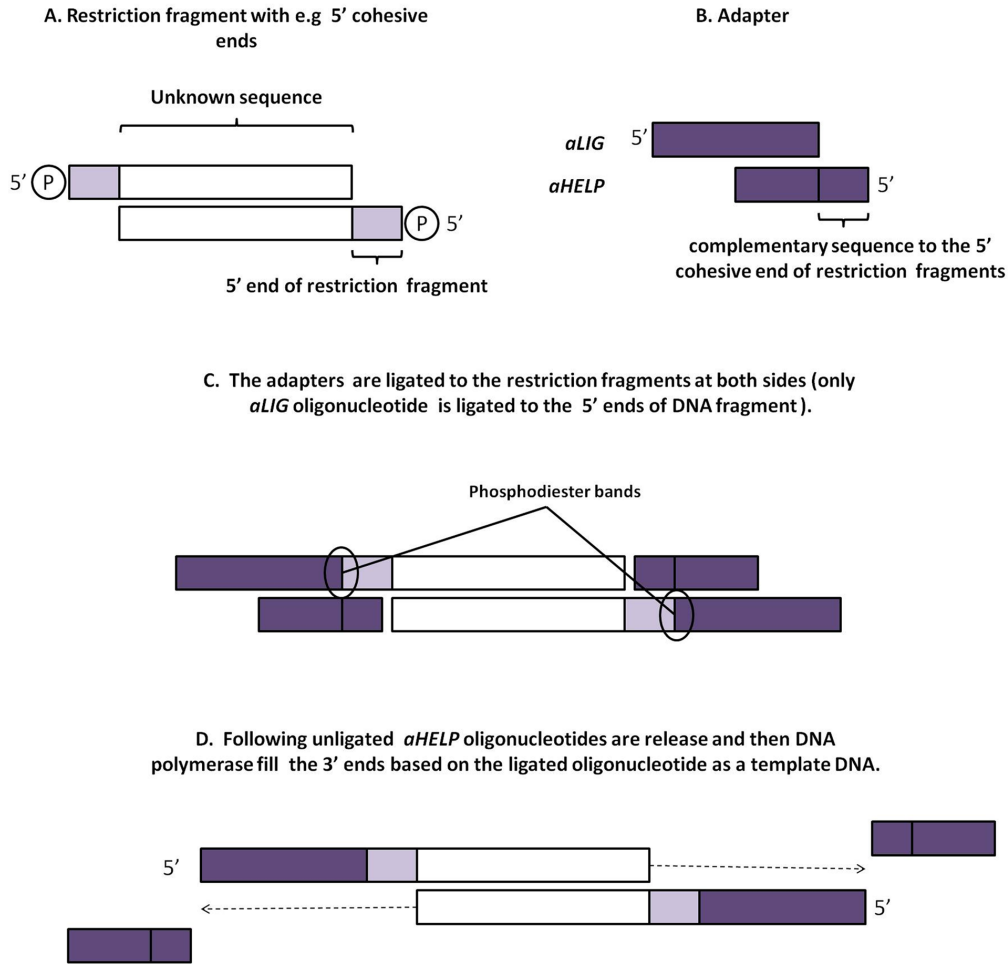

Figure 1. General strategy of LM PCR methods.

graded DNA (50–500 ng) for obtaining readable profiles and for a good reproducibility.

The LM PCR procedure consists of three steps (Fig. 1). In the first step, a total genomic DNA is digested with one or two restriction enzyme(s) giving 5' and/or 3' cohesive ends (Fig. 1A). The enzymes should not be sensitive to DNA methylation. The choice of restriction enzymes depends on the LM PCR variant method. In the second step, double-stranded linkers (synthetic DNA adapters) with complementary sticky ends are ligated to the restriction fragments. The adapter is formed by mixing a helper oligonucleotide (*aHELP*) and appropriate ligated oligonucleotides *(aLIG*) (Fig. 1B). The helper oligonucleotide contains a sequence complementary to the sequence of hanging end of the restriction fragment and it is responsible for leading ends of the double-stranded adapter to the appropriate restriction fragments. The ligated oligonucleotide participates in the formation of a phosphodiester bond with a 5' sticky end generated by restriction enzyme and becomes part of the target DNA in the PCR (Fig. 1C). In the third step, selective PCR is carried out. Each of the LM PCR variant methods differs in the way of selecting fragments to be amplified by PCR. In pre-PCR, dissociation of unligated helper oligonucleotide occurs in initial denaturation step and next a thermostable DNA polymerase fills in the free 3' ends based on the ligated oligonucleotide as a template DNA (Fig. 1D). The adapters (linkers) contain each restriction site sequences and sequences homologous to a PCR primer binding site. Finally, selective amplification by PCR occurs (Figs. 2–6). As a result of PCR, DNA products of different length are obtained and can be visualized after electrophoretic separation as a unique band pattern, or peaks in the case of capillary gel electrophoresis (fingerprint patterns). Differences in these fingerprints between individuals are interpreted as genetic distances.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

The AFLP technique, described for the first time by Vos *et al.* (1995), is based on the detection of genomic restriction fragments by PCR amplification, and can be used for DNAs of any origin or complexity for studying genetic relationships at the species level. The basic AFLP procedure is summarized in a schematic flowchart in Fig. 2. The target DNA is digested with a pair of restriction enzymes (RE1 and RE2, producing three types of DNA fragments (RE1-RE1, RE1-RE2, and RE2-RE2). In most commercial kits for the AFLP method, DNA is subjected to digestion with two different enzymes, one with an average cutting frequency (like EcoRI) and a second one with a higher cutting frequency (like MseI). Since the original publication by Vos *et al.* (1995), several enzyme combinations have been used, such as EcoRI, PstI, HindIII, or ApaI combined with MseI or TaqI (van der Wurff *et al.,* 2000). For this step, it is crucial to ensure complete digestion because incomplete restriction of the DNA causes problems in the AFLP in-

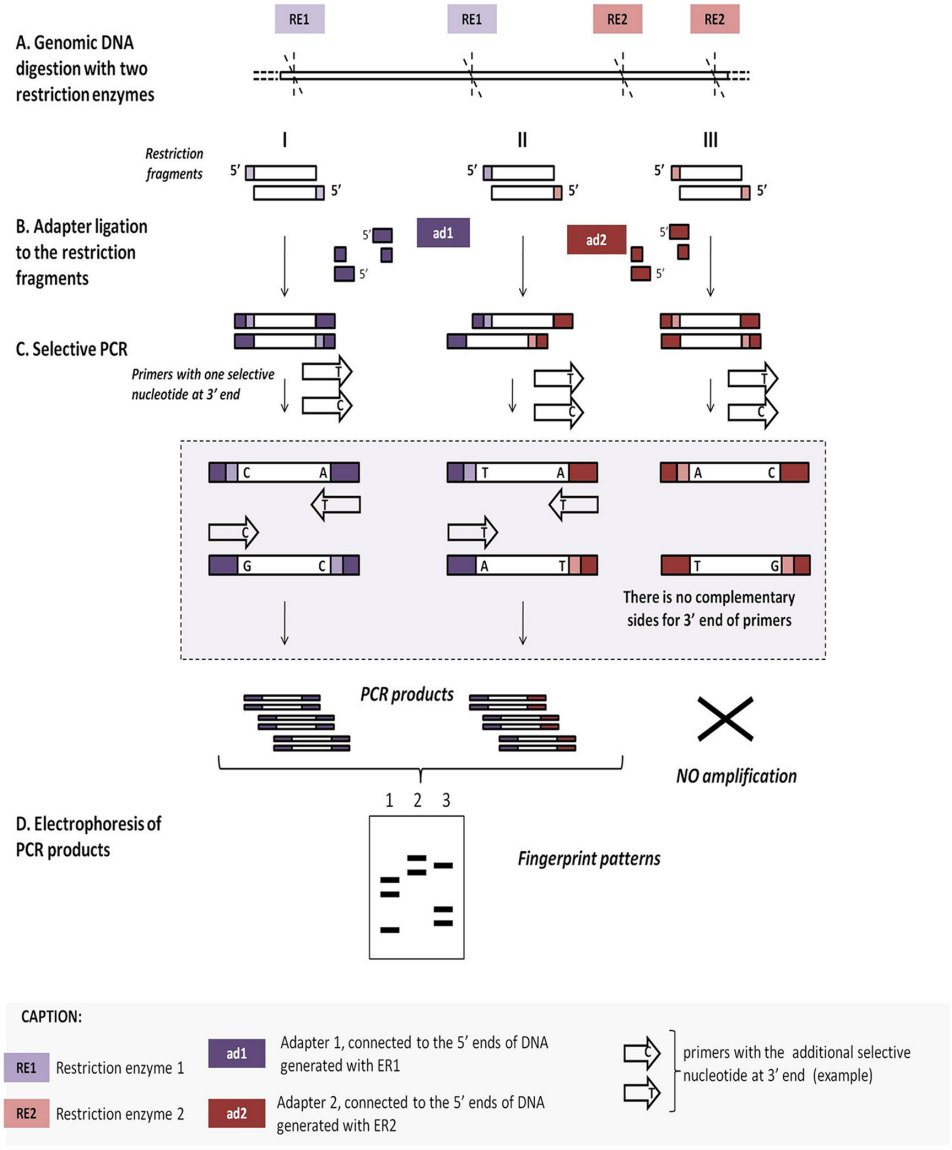

Figure 2. Outline of AFLP method.

terpretation. Amplification of fragments that are not fully digested generates an altered banding pattern and may be misinterpreted as polymorphisms. Next, double-stranded ad1 (for RE1) and ad2 (for RE2) synthetic DNA adapters with complementary sticky ends are ligated to the restriction fragments. Double-stranded adapters consist of a core sequence and an enzyme-specific sequence. For eukaryotic organisms, in the pre-selective amplification, a subset of all the fragments is amplified, using primers that are complementary to the linker sequences with the addition of one nucleotide (A, G, C or T) at the 3'end of the primer. These 'pre-amp' primers will only prime DNA synthesis of fragments with bases flanking the restriction sites that are complementary to the selective nucleotides of the primers, thus reducing the number of fragments to about 1/16 of the initial amount (this can be visualized as a smear when run on an ordinary agarose gel). The number of fragments should be further reduced to a suitable number to be visualized by electrophoresis by a second round of PCR (selective amplification), in which the PCR primers have an additional two to four selective bases at their 3' ends. The number of selective nucleotides on the selective primers should increase with the increasing genome size. By using a combination of several primers (e.g. three primers for small genomes and four primers for large genomes) even single differences between closely related organisms can be detected. For microorganisms (including the eukaryotic ones) no 'pre-amp' is needed. In order to visualize the patterns, one of the PCR primers contains either a radioactive (Dijkshoorn *et al.*, 1996; Janssen *et al.*, 1996; Janssen *et al.,* 1997; Gibson *et al.,* 1998) or a fluorescent (Koeleman *et al.,* 1998) label. Polyacrylamide gel electrophoresis (pattern of bands) or capillary gel electrophoresis (visualized as peaks) with manual or with an automated sequencer are used to detect the subset of amplified fragments. The number of fragments is dependent on the resolution of the detection system. Typically 50–100 restriction fragments are amplified in range size from 60 to 500 bp. An agarose gel electrophoresis may be also used (in such case it is not necessary to label primers) and the patterns may be examined under UV light after staining with ethidium bromide (Gibson *et al.,* 1998) but then fragment length differences of less than 10 nucleotides

Table 1. AFLP method — advantages, disadvantages and advice for users

Advantages	Disadvantages	Advice for users
• offers a relatively fast and inexpensive method for genotyping a large number of individuals with a high degree of resolution and without prior genetic information knowledge • easy, adaptable technique for many studies on plants, animals, fungi and bacteria • regulation of discrimination power by the use of a series of different primer combinations • high discriminative power between and within species (resolution of genetic differences, high stringency), good ability to differentiate clonally derived strains (Dijkshoorn *et al*., 1996; Janssen *et al*., 1996; Janssen *et al*., 1997; Gibson *et al*., 1998; Koeleman *et al*., 1998) • high reproducibility (Dijkshoorn *et al*., 1996; Janssen *et al*., 1996; Koeleman *et al*.,1998) • highly automated, many types of software are available (so reliability is obtained)	• substantial experience of the person carrying out the assay is required • the large number of amplified DNA fragments (e.g. 100) — a software is necessary for analysis • for PAGE, problem with single-nucleotide differences between AFLP fragments • it is the difficult to identify homologous markers • radiolabeled primers reduce the work flow flexibility and AFLP analysis (Hartl & Seefelder, 1998; Huang & Sun, 1999) • capillary electrophoresis requires adjustment and optimization of existing protocols for use without dependency on genome complexity • scoring background noise (Vekemans *et al*., 2002) • lack transparent reporting of error rates (Crawford *et al*. 2012); according to Bonin *et al*. (2004) the error rate per locus has been estimated between 2–5% • existing commercial kits are inapplicable to certain species, especially those with large and complex genomes	• a frequently cutting enzyme with a short recognition sequence rich in A and T may be more appropriate for organisms whose genomes are rich in these nucleotides • the use of the TruI restriction enzyme instead of the commonly used MseI is suggested — a cheaper isoschizomer can produce better quality results (Bensch & Akesson, 2005) • discriminatory power can be changed by replacing the EcoRI enzyme (and the corresponding adapter) with a more frequent cutting enzyme, e.g. TaqI (4 bp recognition site) — an increase of the number of amplified fragments with approximately one order of magnitude • in order to produce easily scored gels you should extend the selective primers by an additional fourth base; it could be useful in combination with an increasing annealing temperature to maintain the target specificity • IRDye® infrared dye (IRD) or other fluorescently labeled oligonucleotide primers instead of radioactive ones, and fragment analysis with an automated DNA sequencing instead of gel electrophoresis improve results

are difficult to score. Many software programs are available e.g. BioNumerics, GeneMapper or GeneScan for the analysis of AFLP patterns. Advantages, disadvantages and advice for user of the AFLP method are shown in Table 1.

Applications of AFLP

- The use in molecular typing in epidemiology, especially in short-term outbreaks (Antonishyn *et al*., 2000; Borst *et al*., 2003; Ball *et al.,* 2004; Fry *et al.,* 2005; Tavanti *et al*., 2007).
- Genome mapping and linkage analyses — AFLPs were used to estimate polymorphism rates and to construct AFLP based linkage maps (Martinelli *et al*., 2005; Kumar *et al*., 2013).
- Markers for diversity, screen of a large number of random sites throughout the genome, thereby generating sufficient data to make comparisons among populations or taxa (Sunnuck *et al*., 2000; Grech *et al*., 2002).
- Phylogenetic reconstructions, bacterial taxonomy (Janssen *et al.,* 1996; Edwards & Beerli, 2000).
- Genetic diversity and population structure, parental analysis comparisons among populations or taxa (Janssen *et al.,* 1996; Edwards & Beerli, 2000; Kumar *et al*., 2013).
- Genetic typing for molecular ecology, evolution (Krauss, 1999; Mueller *et al.,* 1999; Bensch & Akesson, 2005) AFLP band as a locus of specific marker can be isolated — it is possible to extract and sequence the fragment (if electrophoresis is done on polyacrylamide gels) and identify homologous sequences in the database finding markers for genes governing adaptations in wild populations and modifications (Massicotte *et al.,* 2011).
- Measurements of expression variation of multiple genes (cDNA-AFLP) and the distribution of DNA methylation (Bachem *et al*., 1996; Bachem *et al.,* 1998; Xu *et al*., 2004; Salmon *et al.,* 2005).

AMPLIFICATION OF DNA FRAGMENTS SURROUNDING RARE RESTRICTION SITES (ADSRRS)

ADSRRS is a fingerprinting technique based on the digestion of total bacterial DNA with two restriction enzymes differing in cleavage frequency, ligation with two different oligonucleotide adapters and suppression of PCR. PCR suppression (SSH, Suppression Subtractive Hybridization) phenomenon (Diatchenko *et al.,* 1996) constitutes the basis for obtaining a limited representation of the DNA fragments which form the bacterial genome. PCR suppression allows the amplification of only a limited subset of DNA fragments, as only those with two different oligonucleotides ligated at the ends of complementary DNA strands are amplified in the PCR (Masny & Plucienniczak, 2001). The ADSRRS have been described, modified and optimized for genetic typing of microorganisms in epidemiological studies (Krawczyk *et al*., 2003a). The outline of that method is shown in Fig. 3. A genomic total DNA is digested with two restriction enzymes, rare (ERC) and frequent (EFC) cutters. Three kinds of DNA fragments — abundant, sporadic, and limited arise that are formed after digestion with frequent, rare and both cutters at the same time, respectively. The mixture of DNA fragments is ligated with two different synthetic adapters, a short adapter (SAd) and a long one (LAd). All of the 5' ends of the most abundant DNA fragments produced by digestion with a frequent cutter (EFC) are modified by joining with the same long synthetic oligonucleotide. Similarly, sporadic fragments generated by digestion with a rare cutter (ERC) are modified by ligation with the same short synthetic oligonucleotide to both 5' ends of each dsDNA fragment. After filling in of the modifying oligonucleotides joined to the 5' ends with the use of the DNA polymerase, all single-stranded abundant and sporadic DNA fragments have complementary sequences at their 5' and 3' ends (they create the so called "panhandle" structures) and because of that, the proper usage of suppression PCR (SP PCR) during amplification of the genomic fragment mixture should eliminate the most and least abundant DNA fragments from the mixture. The PCR suppression depends on (i) the presence and length of the complementary sequences at the terminal ends of the restriction fragments ligated with adapters (ii) the length of the primer — shorter than an adapter is preferred, (iii) PCR suppression is stronger for short fragments, (iv) PCR suppression is inversely proportional to the concentration of the primers — competition be-

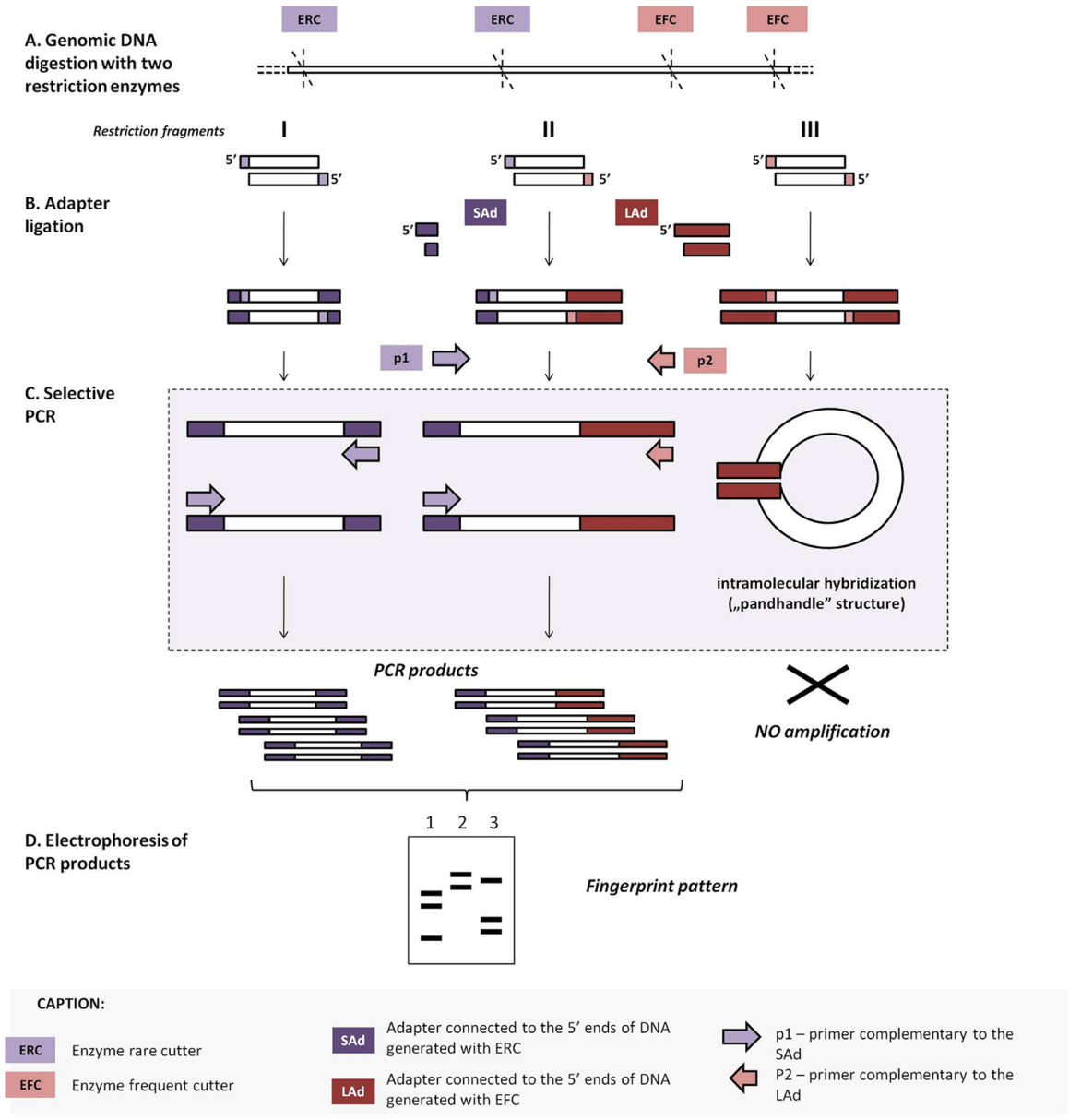

Figure 3. Outline of ADSRRS method.

tween the primer annealing and "panhandle" structure creation.

However, fragments arising after digestion with rare and frequent restriction enzymes at the same time, and consequently joined with two distinct modifying oligonucleotides, are amplified exponentially. After filling in of the modifying oligonucleotides joined to the 5' ends with the use of the DNA polymerase, single-stranded fragments do not have complementary sequences at their 5' and 3' ends, and consequently are not susceptible to SP PCR. Advantages, disadvantages and advice for users of the ADSRRS method are presented in Table 2.

Applications of ADSRRS

The method is useful in epidemiological investigations as was shown for genotyping of many clinically important bacteria such as *Enterococcus faecium* (Krawczyk *et al.*, 2003a), *Serratia marcesens* (Krawczyk *et al.*, 2003b), *Klebsiella pneumoniae* (Krawczyk *et al.*, 2005), *Staphylococcus aureus* (Krawczyk *et al.*, 2007a), *Corynebacterium pseudotuberculosis* (Stefańska *et al.*, 2008), *Campylobacter jejuni* and *Campylobacter coli* (Krutkiewicz *et al.*, 2010).

INFREQUENT-RESTRICTION-SITE AMPLIFICATION (IRS PCR)

The method referred to as infrequent-restriction-site PCR (IRS-PCR) assay (Mazurek *et al.*, 1996) consists of double digestion of genomic DNA with a restriction enzyme that infrequently cuts (RE1) the chromosome giving 5' sticky ends, and a second enzyme that cuts frequently (RE2) giving 3' sticky ends. Restriction fragments produced by digestion with an RE1 enzyme are modified by joining adapters ad1 for which the helper oligonucleotide (*aHELP*) has a phosphate group at the 5' end. But DNA fragments produced by digestion with an ER2 enzyme are modified by joining a synthetic adapter ad2 without a phosphate group, so the helper oligonucleotide (*aHELP*) is removed from this adapter during the denaturation step and this leads to loss of binding sites

Table 2. ADSRRS method — advantages, disadvantages and advice for users

Advantages	Disadvantages	Advice for users
• this method is considered to be reproducible and versatile, and is very useful in studies of clonal relatedness • at least a similar power of discrimination to gold-standard PFGE method (Krawczyk *et al*., 2003a; Krawczyk *et al*., 2003b; Krawczyk *et al*., 2005) • one set of adapters and enzymes can be applied to analyze DNA from diverse species of bacteria • results obtained in PCRs do not depend on the thermostable DNA polymerase (Taq polymerase or Pwo polymerase) or on the thermal cycler used • there are no technical difficulties in implementation of the method under laboratory conditions if a thermal cycler and basic molecular biology techniques are available • the results can easily be analyzed, even on polyacrylamide gels stained with ethidium bromide • PCR products can be directly isolated from the polyacrylamide gel and subsequently sequenced	• this method ensures success only for small genomes • analysis of the series of results of experiments indicates that the source of endonucleases and reaction buffers used for enzymatic digestion of genomic DNA and PCR thermal profile are critical parameters for ADSRRS-fingerprinting methods • lack of an automated system and software • interpretation of the patterns based on the binary system	• it is preferred to use restriction enzymes giving a 5 ' hanging ends with four sticky nucleotides • it is possible to use the restriction enzymes which recognize the different sequences, but giving the same sticky ends after cutting; we can use the same adapter, primer but as a result we obtain different fingerprint, e.g. BamHI or BglII as a frequent cutters enzyme and XbaI or SpeI as a rare cutter enzyme • digestion and ligation reaction may be carried out simultaneously, however to improve the efficiency of ligation it is recommended that it should be carried out separately • ligated oligonucleotide from a short adapter can be also used as a primer in PCR (the same sequence) • the primer sequence should be shorter than linker — this guarantees PCR suppression • a maximum of 19–24 amplification cycles is recommended (more cycles give background noise)

for the primer. The process of amplification of restriction fragments depends on the presence of binding sites for the two primers, as a result of the enzyme selection, adapter's structure and primers. Amplification occurs only for fragments that arose from digestion by both, the rare and frequent cutter enzymes, and the fragments obtained by double digestion by the rare cutting enzyme. The number of DNA fragments generated by IRS PCR depends on the primers used. Additionally, a reduction in the number of amplified fragments is achieved by the addition of one selective nucleotide into the primer. The scheme of the procedure is shown in Fig. 4. Advantages, disadvantages and advice for users of the IRS method are described in Table 3.

Applications of IRS-PCR

This technique has been used, amongst others, in clinical differentiation of isolates belonging to *Acinetobacter baumannii* and *Serratia marcescens* (Yoo *et al*., 1999), *Legionella pneumophila* (Riffard *et al*., 1998), *Staphylococcus. aureus* (Huang *et al.,* 2005), *Klebsiella pneumoniae* (Su *et al.,* 2000), *Coxiella burnetii* (Arricau-Bouvery *et al.,* 2006).

PCR MELTING PROFILES (PCR MP)

The PCR MP technique was developed by Masny and Plucienniczak (2003) and Krawczyk *et al.* (2006) for genetic typing of microorganisms. The scheme of the procedure is depicted in Fig. 5. A total genomic DNA is completely digested with one type of restriction enzyme, such as HindIII or EcoRI for example. All the 5' ends of DNA fragments produced by digestion are modified by joining the same synthetic oligonucleotides. The selectivity and the differentiating power may be adjusted not only by selection of the appropriate restriction enzyme as for AFLP and ADSRRS, but also by changing the denaturation temperature in the PCR cycles. During PCR, all the DNA fragments in the sample should be amplified, but the lowering of the denaturation temperature during PCR should decrease the number of amplified fragments, since only single-stranded DNA molecules may serve as a template in PCR. The PCR MP technique permits a specific gradual amplification of genomic DNA with differing thermal stability, from the less stable DNA fragments, which are amplified at lower denaturation temperature values, to the more stable ones, these being amplified at higher denaturation temperature values. Prior to differentiation of strains by PCR MP, it is necessary to experimentally select the desired denaturation temperature of PCR cycles using a thermocycler with a temperature gradient. By selecting the denaturation temperature of PCR cycles, a decision is made as to the potential differentiation and complexity of band profiles. It is also very important to determine the denaturation temperature range within which the results are reproducible. The denaturation temperature should be optimized separately for each species of microorganisms. For example denaturation temperature range for *Escherichia coli* is 85.0–89.0°C, *Enterococcus faecium* 79.6–82.5°C, *Staphylococcus aureus* 78.3–81.5°C. The selection of the denaturation temperature at which a sufficient number of products could be obtained is very important for reliability of typing results. In this aspect, the versatility of this method is limited. However, the used of the same restriction enzyme, adapter and primer for typing of many micro-

Table 3. IRS-PCR method — advantages, disadvantages and advice for users

Advantages	Disadvantages	Advice for users
• the IRS-PCR technique can be applied to a wide range of bacterial microorganisms while maintaining the same restriction enzymes, primers, adapters and amplification conditions • the use of minute quantities of target DNA • the separation of amplified fragments can be achieved by conventional polyacrylamide gel electrophoresis	• helper oligonucleotide for ends created by rare cutter enzyme should contain the phosphate group which allows to form a phosphodiester bond with restriction fragment — increase in the cost of oligonucleotide synthesis • detection is based on a binary system; it does not take into account the intensity of the bands during detection	• it is necessary to use two restriction enzymes, one of them gives a sticky 5′ end, the second one 3′ ends • frequent cutter enzyme should give the 3′ sticky ends • regulation of discriminatory power can be obtained by choice of the suitable restriction enzymes and by the 3′ end of primer • ligation efficiency increases for >3 bases at 5′ hanging ends

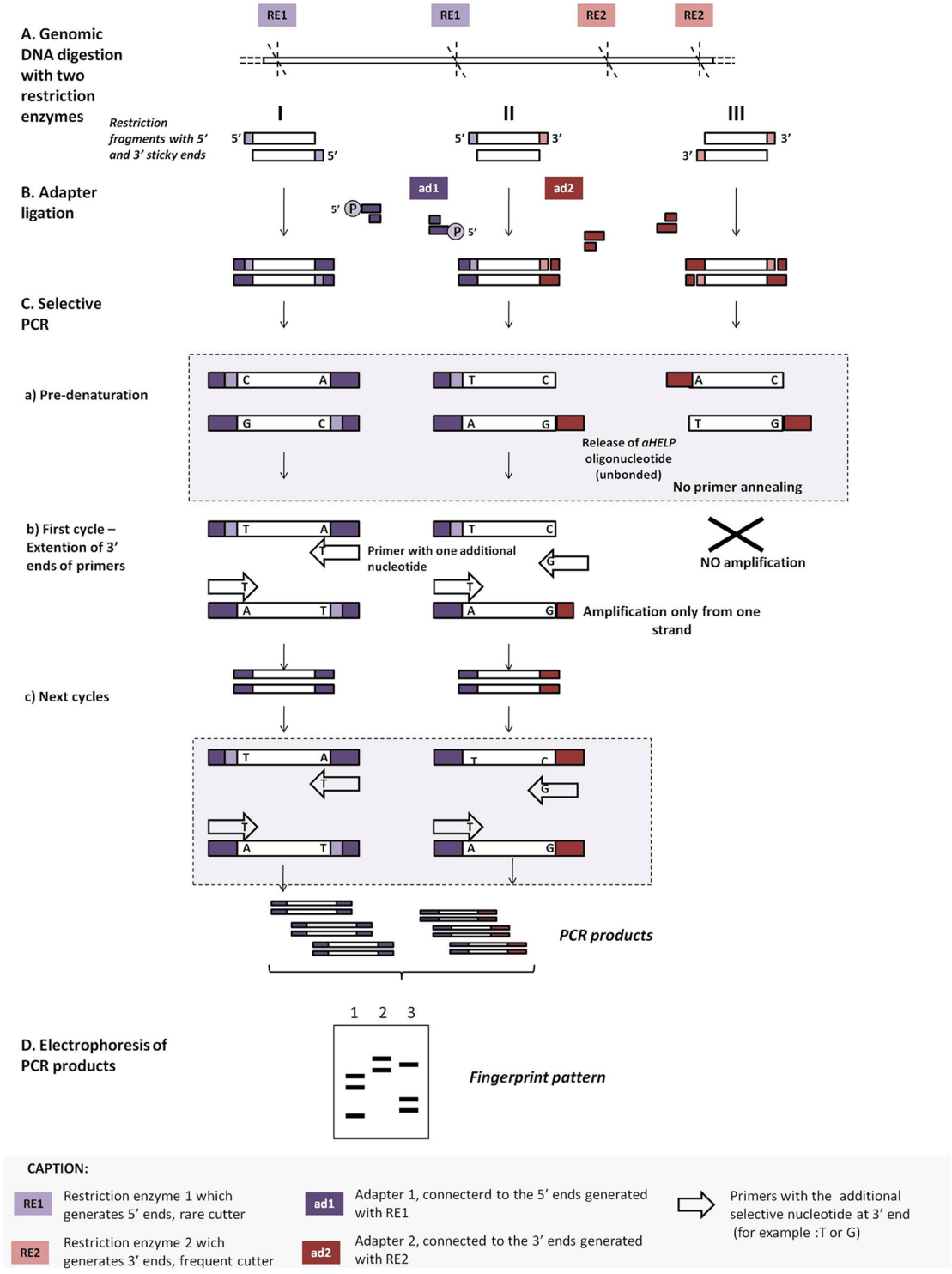

Figure 4. Outline of IRS method.

organisms makes this method flexible. Advantages, disadvantages and advice for users of the PCR MP method are described in Table 4.

Applications of PCR MP

- The use of PCR MP in various aspects of epidemiological studies. The PCR MP technique can be used as an alternative technique for generating DNA profiles in epidemiological studies of intra-species genetic relatedness of bacterial, yeast and dermatophytes strains, similarly to REA-PFGE which is regarded as the "gold standard" in genotyping.
- The possibility of the use in the study of genetic relationships of microbe isolates (bacteria/yeast) obtained from individual patients from hospital wards in order to establish the epidemiological relationship between strains (e.g. associated with their likely transmission from the gastrointestinal tract into the blood system and association with nosocomial infections).
- The possibility of use in a study of bacterial carriers and colonization (Krawczyk *et al.,* 2007a).

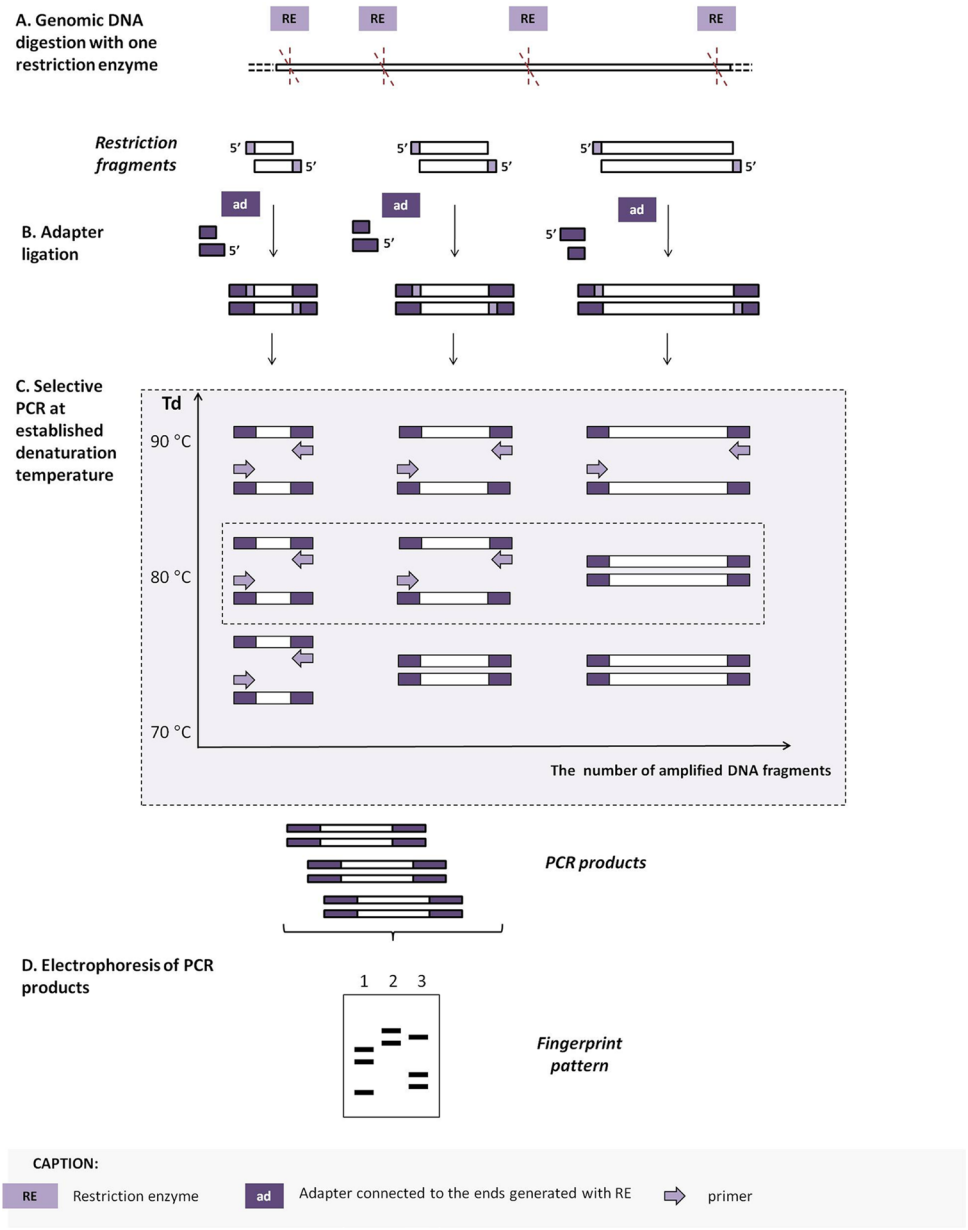

Figure 5. Outline of PCR MP method.

• Verification of the genotyping results for genetically related strains (analysis of the short-temperature gradient PCR cycles) e.g. from short-term episodes (Krawczyk *et al.,* 2006).
• The usefulness of the PCR MP technique in the study of persistent and recurrent bacteremia (Krawczyk *et al.,* 2007b).
• PCR MP has demonstrated suitability for epidemiological examination of *E. coli, Serratia marcescens*, *Enterococcus faecium*, *Klebsiella oxytoca, Candida albicans* (Krawczyk *et al.,* 2006; Krawczyk *et al.,* 2007; Krawczyk *et al.*, 2009; Stojowska *et al.,* 2009a and 2009b) isolates and the method has been applied to the molecular typing of *Staphylococcus aureus* strains (Krawczyk *et al.,* 2007a), *Corynebacterium diphtheriae* isolates (Zasada *et al.,* 2014), *Salmonella enterica* (Zaczek *et al.,* 2015) and to the intraspecies differentiation of *Trichophyton rubrum* and *Trichophyton interdigitale* (Leibner-Ciszak *et al.*, 2010).
• PCR MP bands identified as a locus of a specific marker can be isolated — it is possible to extract and sequence the fragment.
• A method based on ligation-mediated PCR (LM/PCR) using a low denaturation temperature which produces specific melting profile patterns of DNA has been adapted to real-time PCR with high-resolution melting analysis (LM/HRM system) by Woksepp *et al.* (2011). It allowed an analysis of strains isolated from a given outbreak in a single-tube system within a day.

LIGATION MEDIATED PCR/SHIFTER (LM PCR/*SHIFTER*)

The LM PCR/Shifter method relies on the use of a Class IIS restriction enzyme that recognizes nonpalindromic DNA sequences, between 4 and 7 base pairs in length and cuts up to 20 bases outside their target sites, creating different 'sticky ends' up to 5 bases long. Krawczyk *et al.* (2011) used a Class IIS restriction en-

Table 4. PCR MP method — advantages, disadvantages and advice for users

Advantages	Disadvantages	Advice for users
• this method may be used to study DNA from different sources, as well as large genomes, even eukaryotic ones • it is known as a method of universal applicability (does not require specific oligonucleotides for each species) • the discrete number of well-resolved bands generated could readily be analyzed by direct visual comparison • it is possible to verify a questionable genetic pattern through the use of a short-denaturation temperature gradient • it is possible to differentiate DNA fragments of identical length with a different nucleotide composition and this cannot be done by using other LM PCR techniques	• this method requires the use of a thermocycler with a temperature gradient option and is highly sensitive to the denaturation temperature fluctuations • before genetic typing of isolates, it is necessary to implement several preliminary steps, which include: • (i) a regular validation/calibration of the thermocycler; • (ii) the optimization of the DNA denaturation temperature • the fingerprinting pattern is reproducible for a given equipment used, but a fluctuation in band pattern may be observed with different thermocyclers, although the grouping should be maintained (reproducible within the laboratory) • detection is based on a binary system, intensity of the bands is not analyzed	• choice of restriction enzymes — class II, giving about 300–400 fragments (check in silico for the tested species) • the discriminatory power can be regulated by choice of restriction enzymes (e.g. HindIII, EcoRI, SalI, and denaturation temperature values used in PCR • the use of primer complementary to the adapter and 5′ end of the restriction fragment is recommended to avoid the suppression PCR ("panhandle" structure) • the optimal denaturation temperature for the PCR MP procedure should be determined during the optimization experiments with three genetically unrelated strains; analysis of many patterns should lead to finding the most stable denaturation temperature to use • isolates from a particular patient can be tested with PCR-MP at increasing denaturation temperatures — verification of patterns

Table 5. LM PCR/Shifter method — advantages, disadvantages and advice for users

Advantages	Disadvantages	Advice for users
• the simplicity of changing discrimination power achieved by changing the 4 base discriminative sequences in the adapter and/or primer without changing the restriction enzyme used • the PCR conditions can be standardized through the use of common adapters and primers to allow parallel amplification of specific restriction fragments with appropriate adapters without the need to design primers and optimize conditions for each species typing • possible to extract and sequence the fragment that repeats regardless of the 3′ primer sequence • the method is relatively rapid, offering a good discriminatory power and excellent reproducibility (Krawczyk *et al.*, 2011) the results can be easily analyzed, even on polyacrylamide gels stained with ethidium bromide	• a small choice of Class IIS restriction enzymes leaving the 'sticky ends' up to 4 bases long • detection is based on a binary system; it does not take into account the intensity of the bands during detection	• Alw26I restriction enzyme is more useful for typing of bacteria, but FokI is more useful for *Candida* sp. • helper oligonucleotide with 5′ NCGN end and mix primers with 3′ NGCN ends give very good results • the discriminatory power of the method could be changed by 5′ end adapter and/or 3′end primer modification • limited risk of overlapping fragments in a pattern can be obtained by reducing the degeneracy of the primers

zyme such as Alw26I, FokI, giving restriction fragments with different 4 base 5' overhangs. There are 256 possible combinations of the bases at each end of each adapter resulting in 65 536 possible 4-base 5' overhangs (functionally, this equates to 32 768 unique 5' overhangs because of directional reversibility of the 5' overhangs). The ligation of the appropriate oligonucleotide adapters corresponding to the cohesive ends of the DNA fragments, and the selective PCR amplification of ligation products (LM PCR), depending on the 5' end sequence of adapter and 3' end sequence of primer(s) were used. The differentiating potential of the method can be adjusted either by using a mixture of adapters with varying degrees of degeneration (the range of degeneration of these oligonucleotides will cause an increase in the number of amplified fragments) or by combining several primer oligonucleotides in one reaction.

The method facilitates bacterial species strain differentiation on the basis of the different DNA band patterns obtained after electrophoresis in polyacrylamide gels stained with ethidium bromide and visualized under UV light. It may be applied for genotyping studies. The scheme of the procedure is depicted in Fig. 6. Advantages, disadvantages and advice for users of the LM PCR/Shifter method are described in Table 5.

Applications of LM PCR/Shifter

• The method could be especially useful for inter- and intra-species discrimination of microorganism in long- or short-term epidemics, when different discriminatory power might be necessary.

• The usefulness of LM PCR/Shifter method was tested on the *Acinetobacter calcoaceticus-baumannii* complex, *E. coli, Klebsiella oxytoca, Klebsiella pneumoniae, Serratia marcescens*, *Enterococcus faecium* (Krawczyk *et al.*, 2011).

MODIFIED GENOTYPING METHODS WITH THE USE OF LM PCR

For LM PCR methods, the fingerprint patterns do not include any specific information about the species or genus of the tested strain. However, there is a variant of LM PCR for screening, typing and identification of selected organisms e.g. *rec*A-ddLMS PCR (double digestion Ligation Mediated Suppression PCR for *rec*A gene) for *Acinetobacter calcoaceticus-baumannii* complex (interspecific differences) or *rrn*-ddLMS PCR (double digestion Ligation Mediated Suppression PCR for *rrn*-locus) for *Acinetobacter baumannii* (intraspecific typing) (Stojowska & Krawczyk, 2014). The double digestion Ligation Mediated Suppression PCR method is designed to study polymorphism of the sequence upstream from the specific target sequence (species- or genus-specific). In this method only specific restriction fragments containing a genus- or species-specific target sequence are amplified, and it is unnecessary to use genomic DNA isolated from a previously purified bacterial colony.

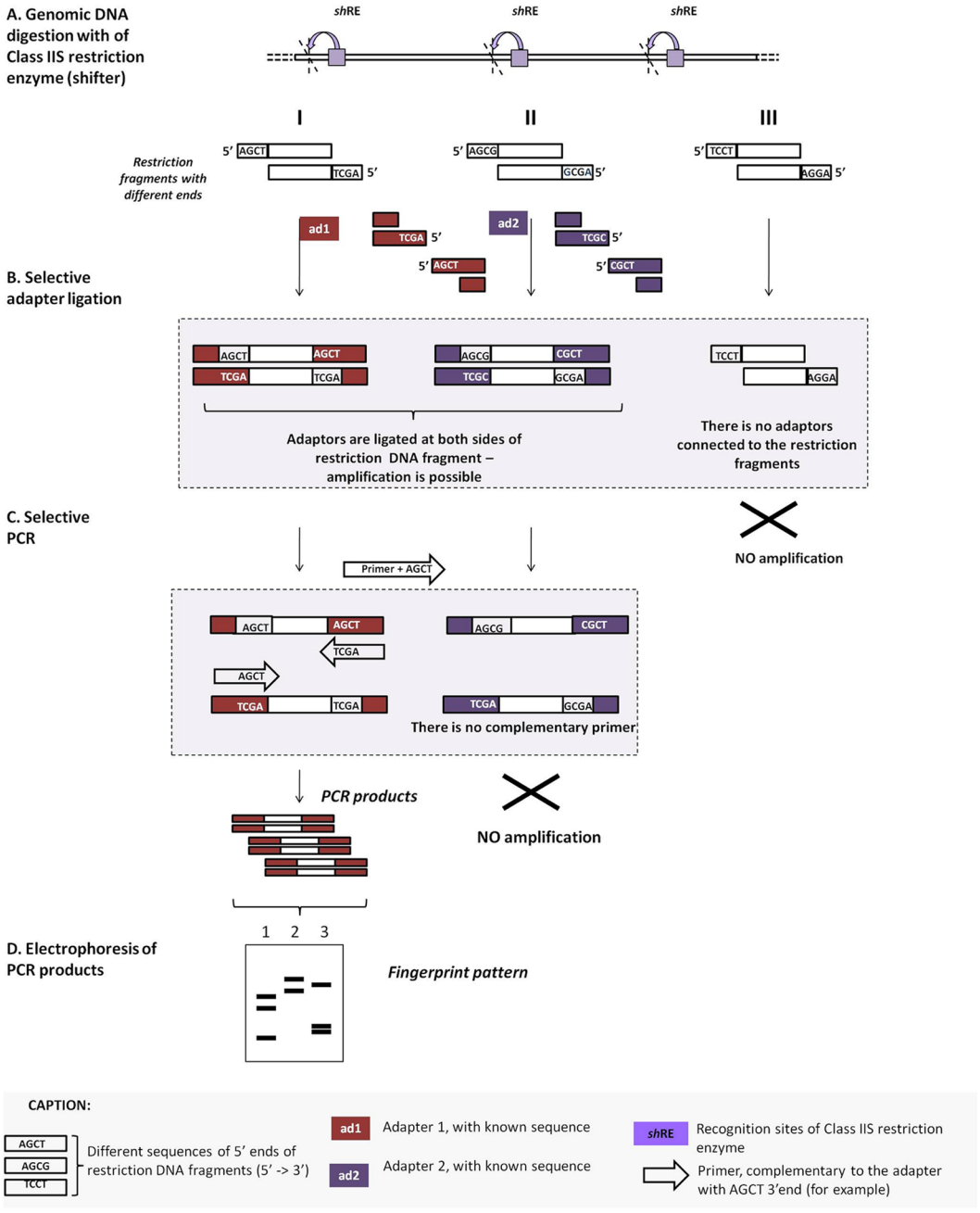

Figure 6. Outline of LM/PCR Shifter method.

Another example of the modified genotyping method with the use of LM PCR is a procedure to amplify the flanking sequences on both sides of the insertion sequence (IS6110) for *Mycobacterium tuberculosis* (Palittapongarnpim *et al.,* 1993). A variation of this method was used by Prod'hom *et al.* (1997) to only amplify the upstream IS6110-flanking region. The other modification of this method was Fast Ligation-Mediated PCR (FLiP) (Reising *et al.,* 2005; Zaczek *et al.*, 2013) and recently developed Fast Ligation Amplification Polymorphism (FLAP) analysis (Zaczek *et al.,* 2014). According to the authors, this method is a specific and valuable molecular tool for analyzing *M. tuberculosis* strains with high discriminatory power as a MIRU-VNTR.

SUMMARY AND CONCLUSIONS

Genomic fingerprinting can give a snapshot of variations at the whole-genome level or focus on smaller amplified genomic regions (PCR-based approaches). This first group of methods is more versatile and simultaneously has a high discriminatory power. The LM PCR technology is a DNA fingerprinting technique that combines both, RFLP and PCR fingerprinting strategies. The comparison of LM PCR procedures is shown in Table 6. These methods are extremely adaptable. Each method has a different system of selection of the amplified fragments (selective primers with additional nucleotides at 3' ends for AFLP, PCR suppression for ADSRRS, lower T_d in PCR cycles for PCR MP, or selective adapters and prim-

Table 6. Comparison of the LM PCR procedures

	AFLP	ADSRRS	PCR MP	LM PCR/Shifter	IRS PCR
Restriction digestion	whole genomic DNA; do not require prior knowledge of an analyzed sequence				
	Two Class II restriction enzymes: a rare and frequent cutter (RC and FC) giving 5′ hanging ends e.g. MseI/EcoRI	Two Class II restriction enzymes: a rare and frequent cutter (RC and FC) giving 5′ hanging ends e.g. BglII/XbaI	One Class II restriction enzyme, a frequent or middle cutter (FC) giving 5′ hanging ends e.g. HindIII	One Class IIS restriction enzyme giving 5′ hanging ends e.g. Alw26I or FokI	Two Class II restriction enzymes giving a 3′ hanging end for frequent cutter (FC) and 5′ hanging end for rare end (RC) e.g. HhaI/XbaI
Ligation reaction with adapter linker	Two adapters (without phosphate group at 5′ end) with complementary overhang to restriction sites	Two adapters (without phosphate group at 5′ end) with complementary overhang: "short" ligated to RT sites and "long" ligated to CT sites	One short adapter (without phosphate group at 5′ end) ligated to restriction sites	Mixed adapters (without phosphate group at 5′ end) with complementary overhang 5′NGCN to 3′NGCN ends restriction sites. 5′adapter ends as a selection factor	Two adapters with 5′ and 3′complementary overhang (with phosphate group at 5′ end for *aHELP* oligonucleotide in case of RC)
Pre-selection PCR	Only for eukaryotic organisms pre-selective primers + 1nt	no	no	no	no
PCR Selection of amplicons	PCR-2 selective primers with 2–3 additional nucleotides at 3′ ends. Selection: reducing the number of amplified fragments is 4^n where "n" is the number of selective nucleotides;	Two primers "short" and "long" Selection: PCR suppresion- panhandle structure for fragments with long primer, the same adapters	Primer is longer than the ligated oligonucleotide with the sequence of restriction site Selection based on the amplification of the unstable DNA fragments by applying a relatively low T_d PCR in cycles;	Selection: depends on 5′ end of adapter and 1-4 selective primers with 3′ NGCN ends	PCR - two selective primers with 1 additional nucleotide at 3′ ends no primer binding sites for FC fragments
	About 50–100 coamplicons	About 10–25 coamplicons	About 10–25 coamplicons	About 10–20 coamplicons	About 10–20 coamplicons
DNA visualisation and analysis	Pre-selective PCR amplification with primer without labelling. Selective PCR amplification with different primers each labelled Capillary electrophoresis	Gel electrophoresis, ethidium bromide, UV light			
Application	For big and small genomes (bacteria, yeast, plants, parasites)	For small genomes (bacteria were the only ones checked)	For big and small genomes (bacteria, yeast, dermathophytes)	For small genomes (bacteria were the only ones checked)	For small genomes (bacteria were the only ones checked)

ers in the case of IRS and LM-PCR/ Shifter) suited to the needs and possibilities of a given laboratory (Table 6). Discriminatory power for each method also depends on the restriction enzyme(s) used at the first step of the method, so it is very important to know the *in silico* established range of cutting frequency for a given species, e.g. for ADSRRS and PCR MP 300–400 cutting sites are recommended. The use of different enzymes can generate a set of different patterns. Polymorphisms detected in DNA fingerprints obtained by restriction cleavage can result from alterations in the DNA sequence, including mutations abolishing or creating a restriction site, and insertions, deletions, or inversions between two restriction sites. The LM PCR methods are simple, affordable, intralaboratory reproducible, with reproducibility comparable to the REA PFGE or better (Krawczyk *et al.*, 2003a; Krawczyk *et al.*, 2003b; Krawczyk *et al.*, 2005; Krawczyk *et al.*, 2006; Krawczyk *et al.*, 2007a; Krawczyk *et al.*, 2007b; Krawczyk *et al.*, 2009), and these advantages make LM PCR techniques a good tool for epidemiological studies (Table 7). Except for AFLP, they neither require substantial experience on the part of person carrying out the assay, nor expensive equipment. Non-degraded good quality DNA is needed, but not as much as

Table 7. Characteristics of the methodology

LM PCR method	Typeability	Quantity of genomic DNA for test (ng)	Intra-laboratory reproducibility	Inter-laboratory reproducibility	Discriminatory power	Versatility	Ease of performance
AFLP	Excellent for all microorganisms	50–500	Good	Good	Excellent	Yes	Good
ADSRRS	Excellent for bacteria	100–200	Excellent	Excellent	High	Yes	Excellent
PCR MP	Excellent for bacteria, yeast, Trichophyton	30–400	Excellent	Good	Excellent	Yes	Excellent
LM PCR/ Shifter	Excellent for bacteria	100–400	Good	nd	High		Excellent
IRS PCR	Excellent for bacteria	100–400	Excellent	Good	High	Yes	Excellent

LM PCR method	Time [h]	Automation	Ease of interpretation	Software	Cost	Availability
AFLP	12	Yes	Fair	Yes	High	Home-made, Commercial
ADSRRS	3	No	Excellent	No	Low	Home-made, Commercial
PCR MP	8	No	Excellent	No	Low	Home-made, Commercial
LM PCR/ Shifter	3		Excellent	No		Home-made
IRS PCR	3	No	Excellent	No	Low	Home-made

for PFGE (permissible alkaline lysis). Detection of amplified fragments is limited to <3000 bp because it depends on the use of polyacrylamide gels. However, these methods can be also performed with agarose gels (e.g. AFLP) with a different range of fragment length, but one should be aware that the sensitivity of detection of amplified fragments will be significantly decreased. These methods are also characterized by a short time of analysis; for ADSRRS, PCR MP, LM PCR/Shifter only 19–24 PCR cycles are required for amplification of restriction fragments. In most cases, it is possible to obtain the typing results within 3–8 hours (after DNA extraction).

Due to the need for digestion of DNA and ligation of the adapters, contamination with foreign DNA coming from the environment is unlikely in spite of the amplification events.

Notwithstanding that, these methods require selection of pure bacterial/fungal cultures and high molecular weight DNA. There are no permanent criteria for the interpretation of the electrophoretic pattern (in epidemiological studies). A pattern-based assay is not easily amenable to interlaboratory standardization and to the development of international databases. These methods can generate both, technical and human errors. Errors can also arise from an incomplete restriction digestion reaction due to the short time of reaction (a solution is to use Fast Digest Enzymes, Fermentas), low activity and possible star activity of enzymes or the use of methylation-sensitive enzymes. The critical parameters are the time required for ligation, the concentration of adapters in ligation reaction and activity of the DNA polymerase in PCR.

In conclusion, LM PCR methods might be very quick methods that are useful in the analysis of microbiological links. They have been used for typing closely related bacteria/yeast/dermatophytes and other microorganisms without knowledge of their nucleotide sequences. Moreover, from a technical point of view, many genomes can be analyzed in a short period of time, and only small amount of DNA is needed. In spite of the fact that reproducibility is often subjected to discussion, they may often be a good alternative tool to differentiate between highly clonal strains.

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