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Review

Methods for the differentiation of microorganisms

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Abstract

Advances in analytical and diagnostic assays based on novel nucleic acid analyses techniques have revolutionized the application of molecular differentiation of microorganisms. Phenotypic typing schemes are now broadly supplemented by new genotyping methods which allow a more refined and detailed differentiation of closely related microorganisms, bacterial strains, isolates and pathogens on the DNA level. Bio-, sero- and phagetyping, antibiotic susceptibility tests, immunoblotting as well as multilocus enzyme- or polyacrylamide gel electrophoresis are now supported by the analysis of plasmid or chromosomal DNA restriction profiles, ribotyping, pulsed-field gel electrophoresis and polymerase- or ligase-chain reaction-based methods or direct sequencing technique to differentiate microorganisms. Some of these molecular techniques are also used in the field of virology to analyse and differentiate closely related sub- or genotypes. Few examples for the analysis and investigation of these usually small genomes will also be given. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The analysis of multiple bacterial isolates by phenotypic or genotypic methods can be used to identify characteristics within a particular species. The division of strains into defined subgroups is called bacterial typing. A prerequisite for all existing typing schemes is the assumption that strains derived from one clone will share certain characteristics in contrast to strains derived from different clones. A bacterial clone might be regarded as an individual. When this bacterial clone divides, the progeny should have an identical genetic composition. However, during the process of multiplication differences at the DNA level can be detected e.g., the exchange of single base pairs, deletion of a gene or even the intake of DNA from other bacteria. These changes are dependent on the number of cell divisions, the genetic distance between strains derived from one clone or on strains of a species and may increase steadily over time [1].

Therefore, strains of the same species isolated from an epidemiological cluster can be usually clearly distinguished from epidemiologically unrelated strains.

Thus bacterial typing has several theoretical and practical implications: (i) to analyse the progress of outbreaks and to examine sequential isolates from a single patient to answer the question whether an infection is new or a relapse, (ii) to associate unusual pathogenic mechanisms with certain strains and (iii) to increase our knowledge of epidemiology of infectious diseases [2].

In order to assess the relatedness of bacterial strains several methods e.g., biochemical reactivity, surface protein composition, enzyme activities, antibiotic sensitivity or a method based on the genetic composition, can be used to determine the degree of similarity.

For the evaluation of typing schemes the three principal and most important issues are: first: a high degree of typeability; second: good reproducibility within one and among different laboratories and third: optimal discriminating capability of the typing system used [1–3]: (1) typeability: the ability to obtain a definite result for each isolate tested; a typing system must give an unambiguous result. (2) Reproducibility: the ability to achieve the same typing result whenever the same strain is tested; typing results should be identical when strains are analysed in duplicate or at different time points. (3) Discrimination: the ability to distinguish between epidemiologically unrelated strains.

The perfect typing method is not available yet, all available methods have certain advantages and disadvantages [3].

Traditional typing techniques based on phenotypic characteristics are increasingly challenged by the use of DNA-based techniques. In the last few years innovative molecular techniques have provided powerful tools for direct DNA analysis. Bacterial typing is an essential implement tool for molecular diagnosis, molecular epidemiology, molecular systematization and molecular archaeology. Bacterial typing is a descriptive discipline based on the subdivision of bacterial species, with the aim to characterize pathogenic bacteria. Molecular typing is based on the natural genetic variation and is the only method available for organisms that can not be cultivated [4,5].

The molecular typing methods e.g., random amplified polymorphism DNA (RAPD), restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE) and sequencing allow to

distinguish bacterial strains formerly classified as one strain (species). Some of the methods described here can not be applied in viral typing schemes, due to the lack of metabolic activities and/or the small genome size.

The most important new perception has probably been the recognition that numerous diseases are caused by a limited number of clonal subgroups e.g., methicillin resistant *Staphylococcus aureus* (MRSA) strains derived from a relatively small number of clones [6].

Detailed analyses demonstrated that quite often defined bacterial clones are the responsible agents. Due to novel and unique combinations of virulence genes or uptake of bacteriophages, plasmids or pathogenicity islands [7], a specific bacterial strain may have a selective advantage to support the selection and spreading of the new variant.

Molecular epidemiology based on genetic relationship has the power to supply a global genetic framework for bacterial classification. Attempts are being made to institutionalize international networks for strain identification e.g., *Mycobacterium tuberculosis* or *Neisseria meningitides* [8,9]. In general, it is difficult to combine data from different studies because each variant needs to be compared and standardized with formerly identified variants.

An enormous advantage is the availability of sequences from whole genomes e.g., *Mycoplasma genitalium*, *Haemophilius influenzae* Rd, *Mycoplasma pneumoniae*, *Helicobacter pylori*, *Escherichia coli*, *Borrelia burgdorferi* sensu stricto [10–15], which will allow the development of typing schemes, reflecting phylogenetic relationship [16].

2. Methods

2.1. Phenotypic methods

The determination of bacterial characteristics is orientated to phenotypic procedures mostly at the level of biologically active entities like protein expression pattern or susceptibility towards infection with certain bacteriophages.

Biotyping is used to monitor biochemical reactivity towards a variety of substrates, whereas antimicrobial susceptibility testing determines the sensitivity or insensitivity towards specific antibiotics. To determine a protein profile of bacteria, serotyping and immunoblotting can be used. Phage typing investigation gives answers concerning the susceptibility to bacteriophage infection. Multilocus enzyme electrophoresis reveals allele variability of certain key enzymes. In general traditional phenotyping methods have two major disadvantages: first of all they are limited to the restricted number of characteristics that can be examined and second they might generate misleading results due to the variable alterations in gene expression [17,18].

2.1.1. Biotyping

A panel of biochemical reagents can be used to identify an organism by its reaction and classify it on the genus and/or species level. The fermentation of glucose, lactose or other sugars may be easily detected by the production of gas or change in color of an indicator dye caused by the production of acids. The pattern of utilization of carbon sources plays an important role in the differentiation of species. The most obvious disadvantage of biotyping is, that it can not be used for the discrimination of single isolates within one species and is therefore not suitable for the typing of bacteria.

2.1.2. Antibiotic susceptibility

The antimicrobial susceptibility (antibiogram) is analyzed by the growth of an isolate in the presence of a given antibiotic. The antibiogram, although the most standardized typing method, is not very discriminatory. Furthermore, antibiotic susceptibility patterns can change rapidly e.g., transformation of plasmids containing resistance genes. Only in individual cases antibiotic susceptibility can be used as a typing procedure [19].

2.1.3. Phage typing

Phage typing has been extensively used to analyze the epidemiology of *Staphylococcus aureus* and *Salmonella* ssp. [20]. Phage typing is not very discriminatory and therefore only of limited use.

2.1.4. Serotyping

The epidemiology of several bacteria e.g., *Legionella pneumophila*, *Streptococcus pneumoniae*, *Escherichia coli* O157:H7 and *Borrelia burgdorferi*

sensu lato [21–26] has been extensively investigated using serotyping. Like phage typing this method is not very discriminatory and therefore restricted to certain applications.

2.1.5. Polyacrylamide gel electrophoresis (PAGE)/ immunoblotting

Whole cell protein pattern (Fig. 1) can be used for typing and classification of different strains [27,28] and has been described for epidemiological investigations of several bacteria e.g., *Staphylococcus aureus*, *Clostridium defficile* and *Borrelia burgdorferi* sensu lato [29–33]. Immunoblotting can be used to analyze antigenic variations, strain heterogeneity of different bacteria species and also to discriminate viral subtypes (Fig. 2; HIV-Immunoblot).

2.1.6. Multilocus enzyme electrophoresis (MLEE)

Water soluble cellular enzymes are separated on starch gels or cellulose acetate sheets by electrophoresis followed by staining of various enzymes [34].

Differences in the amino acid sequence can lead to differences in the mobilities of enzyme variants. Each unique combination of protein variants is referred to an electrophoretic type (ET) and is compared with a wide range of reference strains. Standardized MLEE classification for studying populations of bacteria has attained international acceptance for *E. coli*, *S. enterica* and partly for *Neisseria meningitidis* [35–37].

The disadvantage of this method is the limited applicability for epidemiological analysis of clinical isolates, as shown for virulent *E. coli* isolated from patients with pyelonephritis, which revealed the same MLEE pattern although they were epidemiologically unrelated [38]. MLEE is generally more useful to identify species rather than to discriminate isolates of the same species [3].

2.2. Genotypic methods

In contrast to the phenotypic methods, the genotypic methods directly analyze the DNA. The obvious advantage of these methods – like chromosomal DNA restriction profiles, nucleic acid probes, ribotyping, pulsed-field gel electrophoresis, PCR and sequencing – is the independence of restricted numbers of organism characteristics.

In general, the genotypic procedures are consid-

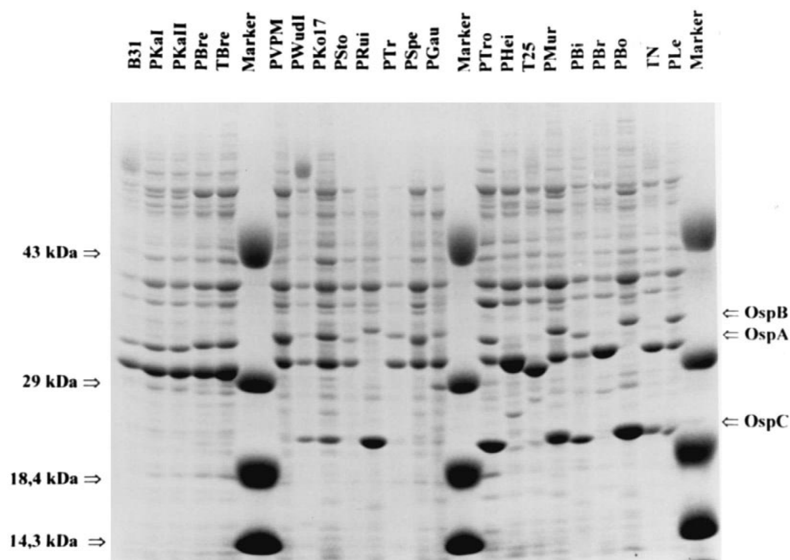

Fig. 1. Protein separation by SDS-PAGE. Protein from whole-cell lysate of different *Borrelia burgdorferi* sensu lato isolates were subjected to 10–20% SDS polyarylamide gradient gel electrophoresis using a standard procedure [32]. Protein bands were visualised by staining with Coomassie blue. Outer surface proteins (Osps) A,B,C, as indicated are important surface proteins.

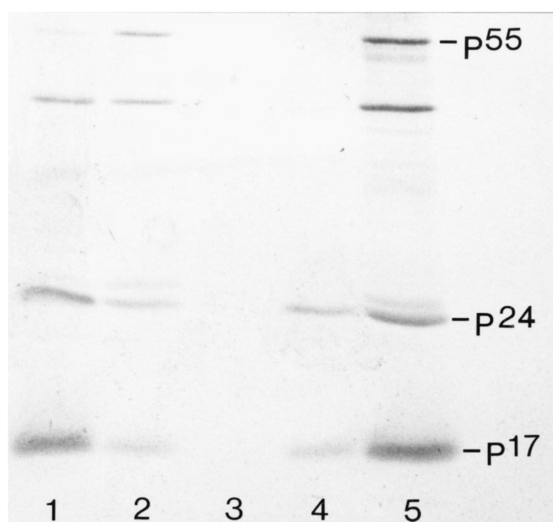

Fig. 2. Immunoblot of HIV-1 particles released from virus-infected lymphocytic cells. The viral proteins were analyzed after separation on a SDS-polyacrylamide gel using antibodies against the viral structural proteins p17 and p24. The viral protease cleaves a p55 gag-precursor protein into the mature structural proteins p17, p24 and p15 [127]. Two viral populations different in the processing capacity of the protease were investigated. The active wild-type enzyme (lanes 1+4) cleaves the p55 precursor efficiently into the mature products p24 and p17, whereas the altered enzyme is less active as indicated by the large amounts of unprocessed p55 precursor compared to the cleaved product (lanes 2+5). Lane 3: non-infected control. The virus with the altered enzyme reveals a less aggressive phenotype with reduced propagation capability in cell culture.

ered to be technically more complex; but typeability, reproducibility and discrimination power revealed better results [1]. Another obvious advantage is the general use for any type of bacteria including bacteria which can not be cultured.

2.2.1. Plasmid profiles

Plasmids are self-replicating autonomous extrachromosomal DNA elements. The reliability of this method depends on the presence of plasmids in the isolates to be investigated. Analysis of plasmid profiles is an interesting way to compare the identity of isolates and a wide range of species was already typed with this method [39–44]. For example, this method is used to characterize and group *Borrelia burgdorferi* sensu lato within a given species and in particular to determine the plasmid content of an isolate. *B. burgdorferi* sensu lato has supercoiled circular plasmids [45,46] and linear plasmids [47]. These linear plasmids are considered as a possible useful typing tool in the classification of *B. burgdorferi* sensu lato isolates (Fig. 3) [48–51].

2.2.2. Chromosomal DNA restriction profiles

For this type of analysis chromosomal DNA is digested by restriction endonucleases and the generated fragments are separated by agarose gel electrophoresis.

Restriction endonucleases are enzymes cutting DNA at a defined position within (or close to) a specific recognition sequence. Since restriction endonucleases are highly specific, complete digestion of a given DNA provides reproducible pattern of DNA fragments whereby the number and size of fragments depends on the DNA composition. Variation in the pattern of fragments is called restriction fragment length polymorphism (RFLP) and can result from a

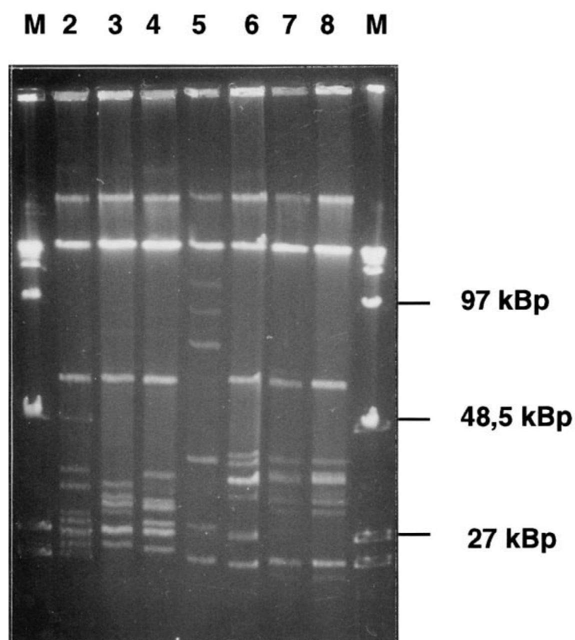

Fig. 3. Linear plasmid profiles of different *Borrelia burgdorferi* sensu lato isolates. The *Borrelia* strains were embedded in agarose blocks, lysed with lysozyme and digested with proteinase K. For plasmid profile analysis, agarose sheets were used without further treatment. The analysis was done with pulsed time of 0.9 to 2.5 s for 30 h. Lambda concatemers with a monomer size of 48.5 kbp and marker II (Boehringer Mannheim, Germany) were used as length marker.

minor change of DNA e.g., single base substitution within the restriction enzyme recognition and/or cleavage site, or major changes like insertions, deletions or sequence rearrangements. A limitation of restriction enzyme digestion of whole chromosomes is the generation of a large number of different fragments. The separation by conventional gel electrophoresis leads to a smear-like appearance of the DNA on the gel and the yielded band pattern are also too complex to be properly compared. To master this problem, the method was combined with nucleic acid probes.

2.2.3. DNA restriction profile with nucleic acid probes

In order to decrease the number of fragments resulting from DNA-RFLP nucleic acid probes can be used to compare the yielded pattern more easily. The restriction fragments are separated by agarose gel electrophoresis, transferred to nitrocellulose or nylon membranes by southern blotting [52] and hybridized with a labeled probe. This probe, derived from insertion sequences, based on specific virulence factors or being randomly cloned, is labeled with a highly specific detector group. The probe consists of fragments of single-stranded nucleic acids (mostly DNA) binding to complementary target nucleic acid sequences, whereas the lengths of this fragments range from 15 to 10 000 nucleotides. Only the genomic DNA restriction fragment that is able to hybridize to the probe will be visible after staining, thus greatly simplifying the fingerprint.

2.2.4. Probes derived from insertion sequences

Insertion sequences (ISs) are transposable repetitive DNA elements, usually present within bacterial genomes in numerous copies. Bacterial IS elements are compactly organized, with a typical single coding sequence consisting of short identical or nearly identical sequences at both ends opposite in orientation and have a size of usually 9 to 40 bp. The IS elements are typically less than 1500 bp long (e.g., the IS-element of *Mycobacterium tuberculosis* IS986 is 1358 bp long [53]). The discrimination ability of this method depends on the number of copies of the IS element in the bacterial genome. The differentiation of *Mycobacterium tuberculosis* by IS6110 has been standardized [8] and distinguishes reliably different strains of this species. In contrast, only a poor ability of distinction is described for isolates with less than five copies [54].

2.2.5. Ribotyping

Ribotyping (ribosomal DNA-RFLP analysis) assesses the RFLPs within the genes coding for ribosomal RNA (16S and 23S rRNA), sequences which are highly conserved. The genes for rRNA are present in several copies in the bacterial chromosome and thus hybridization with probes for rRNA reveals only chromosomal fragments containing the rRNA gene sequence. The number of hybridization bands (usually 7 to 12) is small enough to allow simple analysis of the banding pattern. Grouping of bacteria on the basis of this method is called ribotyping. Ribotyping has been frequently used for subserogroup characterization of bacteria belonging to different species [55], whereas isolates from one outbreak typically share the same ribotype. The method can be used for long-term epidemiological studies since the ribotype is stable after in vitro and in vivo passages [56]. The discriminatory power of ribotyping is lower compared to PFGE [57–59], but can be increased by using two different restriction enzymes [60]. For *Salmonella* serotype typhi ribotyping may be the only subtyping method that offers sufficient discrimination potential to be used in epidemiological investigations [61,62]. For bacteria species containing only one or two copies of genes coding for ribosomal RNA (*Mycobacterium* spp. and *Mycoplasma* spp.) ribotyping is not favorable.

2.2.6. Pulsed-field gel electrophoresis (PFGE)

For separation of DNA molecules conventional agarose gel or acrylamide gel electrophoresis is the most frequently method used. However, its applicability is restricted to molecules <50 kilo base pairs (kbp), as electrophoretic mobilities become increasingly independent of molecular size [63]. To overcome this limitation the PFGE technique was developed [64–67]. This technique allows to resolve karyotypes, chromosomes of different size and mapping of chromosomes for many bacteria.

Intact cells of bacteria are embedded in agarose in order to protect the DNA during the subsequent extraction. The agarose blocks are then treated with detergent and enzymes to isolate the DNA. The

embedded chromosomal DNA is cut with a restriction endonuclease that recognizes only few sites to create only a small number of fragments [68]. The resulting fragments (10–800 kbp) can be separated in an electric field with the angle of migration repeatedly switching.

The molecules are subjected to electric fields applied alternatively in two directions. This switching allows smaller fragments a more rapid orientation in contrast to longer molecules; therefore smaller fragments migrate faster than larger ones and the DNA fragments can be efficiently resolved. Today, PFGE is the most common used epidemiological tool [68] and it is often an optimal typing method for molecular epidemiology [69]. Many different bacteria species (Fig. 4) were analyzed by this technique [51,68,70–79]. A suggestion to standardize the interpretation of the results has been published [68].

PFGE is also a suitable tool for the determination of species of *Borrelia burgdorferi* sensu lato strains (Fig. 5) and allows further characterization by LRFP [51,72,74,80].

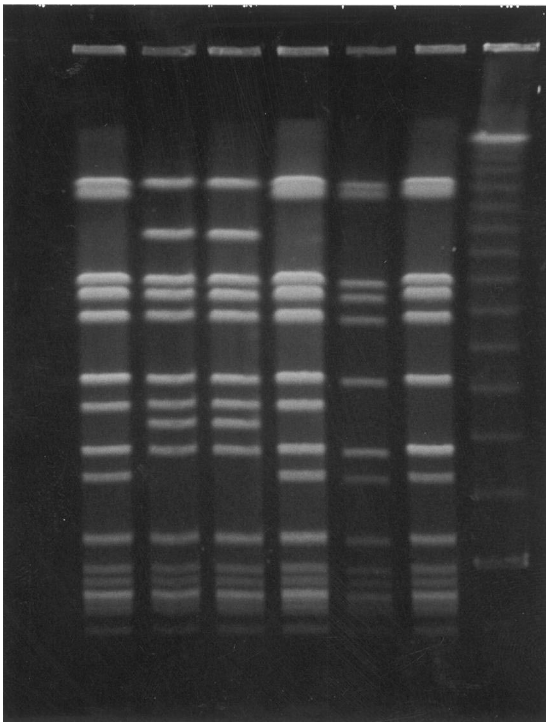

Fig. 4. Pulsed-field gel electrophoresis of methicillin resistant *Staphylococcus aureus* (MRSA) strains. For sample preparation see Fig. 3. The embedded *S. aureus* DNAs were digested with *Sma*I and separated with pulse times from 1 to 45 s for 22 h.

In contrast to SDS-PAGE and immunoblotting, the PFGE analysis exhibited a distinct heterogeneity within the species *Mycoplasma hominis* (unpublished results). The choice of the appropriate restriction enzymes depends on the DNA base composition of the organism. For the G+C poor *M. hominis* strains (G+C<30%), restriction enzymes with recognition sequences rich in G+C were chosen to yield only a few fragments. The two restriction enzymes *Sma*I and *Mlu*I are suitable for this kind of investigation, since both restriction enzymes yield four to eight fragments with appropriate sizes mentioned above. The application of restriction enzyme cleavage patterns analyzed by PFGE is a suitable and easy method to demonstrate hetero- or homogeneity of isolates within one diagnosis group without the need for subsequent hybridization using various probes [51,74].

2.2.7. Polymerase chain reaction (PCR)

Today, polymerase chain reaction (PCR)-based methods are used for bacterial genotype identification and discrimination of bacterial strains. The PCR technique is based on repeated cycles of high temperature for denaturation of the DNA, oligonucleotide (primer) annealing and an extension step which is mediated by a heat stable polymerase. In each cycle of the PCR the number of copies of the chosen sequence is doubled so that the amount of the target DNA is exponentially increasing. Usually the target DNA is separated by agarose gel electrophoresis and stained with ethidium bromide. Amplification of 16S rRNA with different species-specific primer pairs (Fig. 6) allows identification of the three pathogenic *Borrelia burgdorferi* sensu lato species [51,81]. An important tool for genotyping is the sequencing of PCR-amplified 16S rRNA and comparison of the data with sequences from the taxonomic databases. This approach is required for identification of bacteria that can not be cultivated.

Analysis by intergenic spacer length polymorphism e.g., 16S-23S rDNA spacer or inter-tRNA spacer length polymorphism is a suitable tool for genotypic characterization [82,83]. By using a multiplex PCR within a single PCR assay several regions of species can be determined. Multiplex PCR is a

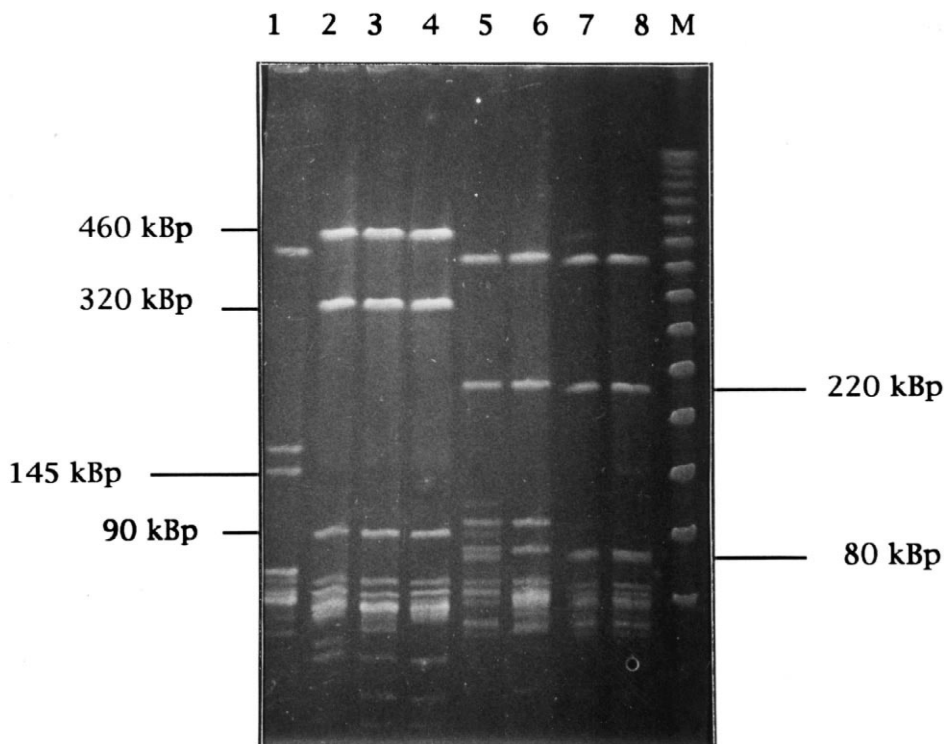

Fig. 5. Pulsed-field gel electrophoresis of *Borrelia burgdorferi* sensu lato digested with *Mlu*I. The large restriction fragment pattern (LRFP) after *Mlu*I digestion of different *Borrelia burgdorferi* sensu lato strains showed three characteristic bands with sizes of 460, 320 and 90 kbp for *B. afzelii* (lane 2 to 4), two characteristic bands with sizes of 220 and 80 kbp for *B. garinii* (lane 5 to 8) and one specific band with a size of 145 kbp for *B. burgdorferi* sensu stricto (lane 1).

combination of multiple primer sets for the parallel amplification of different targets in one reaction [84–86]. As an example this method has been used for simultaneous detection of Hepatitis A virus (HAV)- and Hepatitis C virus (HCV)-RNA (Fig. 7).

For detection of sequence polymorphism in DNA fragments of several hundred base pairs the single-strand conformation polymorphism (SSCP) analysis can be used [87]. As an example for an SSCP-application the differential analysis of various HCV genotypes is shown in Fig. 8.

2.2.8. PCR-restriction fragment length polymorphism (RFLP)

PCR amplicons are often further characterized by subsequent restriction with different enzymes to yield defined restriction pattern due to sequence variation within the amplified DNA fragment (PCR-RFLP) [88–90]. As an example the discrimination of various Hepatitis C virus genotypes by this method is shown in Fig. 9.

2.2.9. Repetitive chromosomal elements (REP)-PCR

Repetitive chromosomal elements (REPs), which are found in all bacteria randomly distributed in genomes, are the targets of this specific PCR-based method. Primers anneal to repetitive chromosomal element sequences and a DNA fragment is amplified if such sequence elements are located closely enough to each other to support efficient amplification between neighboring primer binding sites. The number and sites of these repeated sequences are variable from strain to strain, therefore a different pattern will be generated. A typical REP-PCR is the ERIC-PCR (enterobacterial repetitive intergenic consensus PCR), based on conserved sequences in Enterobac-

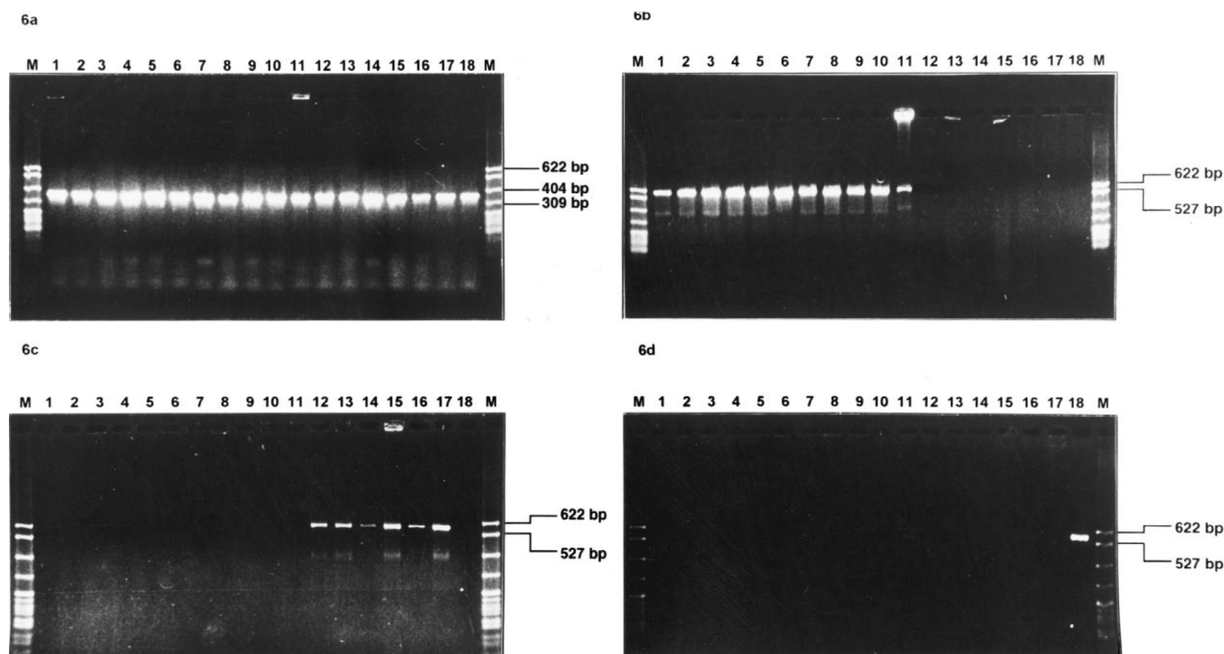

Fig. 6. 16S rRNA-specific PCR. As a target for PCR amplification, the gene coding for 16S rRNA was selected. With the *B. burgdorferi* sensu lato primers a specific 357 bp amplicon was generated from all isolates (see a). Eleven strains revealed an amplicon of 574 bp only with the *B. garinii* specific primers (b, lanes 1 to 11) – these strains were identified as *B. garinii*. Six strains were identified as *B. afzelii* by an amplicon of approximately 591 bp only with a specific primer pair for *B. afzelii* (c, lanes 12 to 17). With the specific primers *for B. burgdorferi* sensu stricto only one strain showed an amplicon with approximately 574 bp and was identified as *B. burgdorferi* sensu stricto (d, lane 18).

teriaceae [91,92]. Similar techniques for eukaryotes have also been described e.g., Alu-PCR [93].

2.2.10. Random amplified polymorphism DNA (RAPD) or arbitrary primed (AP)-PCR

Arbitrary primed PCR (AP-PCR) or the random amplified polymorphism DNA (RAPD) method [94,95] were used to type a wide range of bacteria [78,96–105]. AP-PCR uses primers with a length comparable to those used in standard PCR (18–24 bp), whereas RAPD uses shorter primers (typically 6–10 bp). Both methods are less useful to differentiate small genomes (such as those of viruses), since the number of products is usually small. Arbitrarily selected short primers are used to anneal with low stringency at multiple loci on the chromosomes. These short primers will hybridize at random sites on the chromosome and if the sites are located nearby an amplicon is generated. The DNA fingerprints differ accordingly to the degree of relatedness of the strains investigated. Problems were described with the reproducibility and difficulties occur in comparing the different patterns [2].

2.2.11. Ligase chain reaction (LCR)

In contrast to the above described PCR techniques, the ligase chain reaction (LCR) uses the enzyme DNA-ligase. The main difference to other polymerase-based nucleic acid amplification methods is the fact, that no new synthesis of DNA is required. Repeated cycles of oligonucleotide hybridization and joining generates multiple copies of the target sequence. For application of LCR [106] two complementary pairs of oligonucleotides hybridize to the target DNA. After ligation, the target–ligation product duplex is separated by thermal denaturation and is then available as new template for the hybridization and ligation of probes during the next cycle. Therefore, in each cycle of oligonucleotide hybridization the number of templates is doubled. The LCR

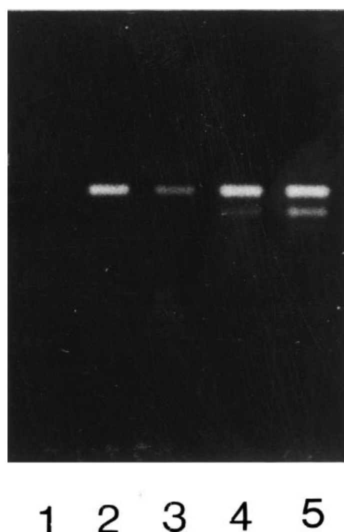

Fig. 7. Simultaneous detection of Hepatitis A virus (HAV)- and Hepatitis C virus (HCV)-RNA by multiplex PCR. RNA of both viruses was extracted, copurified and then reverse transcribed using two specific oligonucleotides each specific for one virus in a single tube reaction. The obtained cDNAs were used to perform ''nested'' PCR with two sets of primers for each virus in one reaction. The products of the second PCR-amplification were analysed on a 2% agarose gel in TBE buffer. PCR amplicons were stained with ethidium bromide and visualized under UV light. Lane 1 (negative control): primers for HAV and HCV were added, no specific viral RNAs detected. Lane 2 (HCV-RNA positive sample, no HAV-RNA present), only HCV-specific primer added: detection of a 283 bp amplicon derived from HCV-RNA, no HAV-RNA amplicon. Lane 3 (HCV-RNA positive sample, no HAV-RNA present), HCV- and HAV-specific primer added: detection of a 283 bp amplicon derived from HCV-RNA, no HAV-RNA amplicon. Lanes 4 and 5 (HAV/HCV-RNA positive sample, HCV- and HAV-specific primer added: detection of two PCR amplicons, the upper fragments derived from HCV-RNA and with the same molecular mass as the amplicons in lanes 2 and 3 (283 bp). In addition, the smaller (242 bp) HAV-specific amplicons are detected [128].

method is used for detection of virulent poliovirus revertants in vaccinations, human papillomavirus, human immunodeficiency virus-type 1 (HIV-1) and *Mycobacterium tuberculosis*, as well as for genetic disease diagnosis [107–110].

2.2.12. Sequencing

The determination of the nucleotide sequence of multiple genes from numerous strains is reliable and simple to interpret, but requires a lot of technical competence. Sequence analysis might become increasingly important for typing, if this method can be further simplified to be performed on a routine basis in laboratories which carry out epidemiological or genotype studies [1].

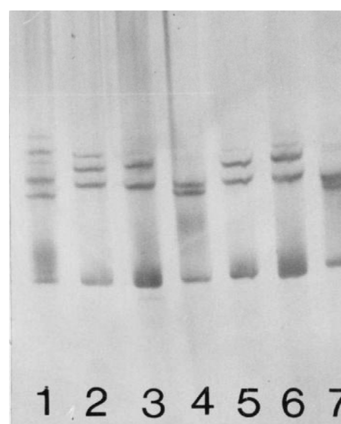

Fig. 8. SSCP analysis of different HCV genotypes. A 283 bp DNA fragment from the 5′-non translated region of the virus is amplified by RT-PCR (see also Fig. 7). The amplicons are analyzed on a 7.5% polyacrylamide–5% glycerine gel; PCR products of different HCV genotypes are separated due to differences in their secondary structure. Lane 1: genotype 3a; lanes 2 and 5: genotype 1a; lanes 3 and 6: genotype 1b; lanes 4 and 7: genotype 2b. In contrast to the RFLP analysis in Fig. 9 the closely related genotypes 1a and 1b cannot be differentiated using this technique.

A new described sequencing method, the multilocus sequence typing (MLST), was carried out by sequencing six gene fragments (house keeping genes) with about 470 bp. The great advantage is the portability of sequence data, which allows laboratories of different countries and continents to submit the sequence data from the house-keeping gene fragments to a central World-Wide Web site containing the database for that species [9]. In summary, so far there are not yet enough data available to confirm, that this method will be suitable in the near future.

3. Conclusions

In general, typing systems can be subdivided into two separate groups: phenotypic and genotypic methods. Phenotypic techniques are designed to detect

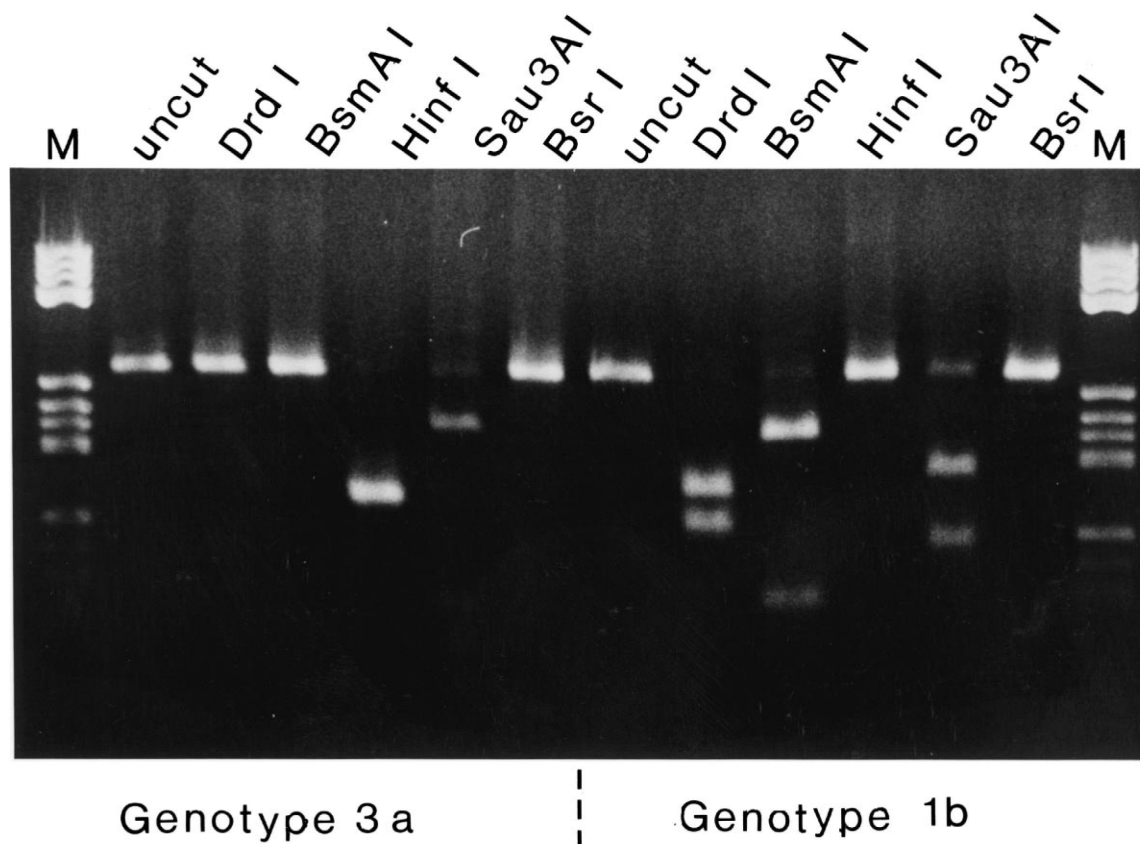

Fig. 9. RFLP analysis of two different Hepatitis C virus (HCV) genotypes. A 283 bp DNA fragment from the 5′-non translated region of the virus is amplified by RT-PCR [129] and cleaved with five different restriction enzymes. The restriction products are separated on a 1.5% agarose gel according to their length, stained with ethidium bromide and visualized under UV light. Left side: genotype 3a derived PCR products cut with the enzymes indicated; right side: genotype 1b derived products. M: Marker DNA used as length standard. The clinical importance to perform HCV genotyping is based on the assumption that patients infected with certain genotypes respond more effectively to interferon-alpha treatment than others [130–134].

features exprimed by bacteria whereas genotypic methods are based on the DNA-level. Some of the classical phenotyping methods are biotyping, serotyping, lysotyping, antibiotic-resistance testing, electrophoretic protein separation, immunoblotting and multilocus enzyme electrophoresis. For example, a sensitivity of 95% in diagnosis of acute gonococcal urethritis in men has been achieved by the examination of Gram-stained smears [111], but this method is not suitable to differentiate between two strains of the same species. Profit and possible use of these techniques are limited by the appearance of phenotypic differences of isolates of the same strain. Beyond that, few of the strains cannot be standardized as the features to be tested are not detectable.

Therefore, classical phenotypic methods of standardization are often not suitable to yield a satisfactory typing analysis.

For that reason, typing methods are used to detect differences on the DNA level, such as ribo-standardization, plasmid-profile analysis, restriction endonuclease analysis, PCR and PFGE. These methods proved to be exceptionally helpful in typing analysis to answer questions concerning infection chains and comprehension of evolution of pathogenicity.

The natural genetic variation in chromosomal

DNA of one bacterial species enables molecular typing systems to distinguish unrelated strains. The higher the variation, the fewer the problems to distinguish unrelated strains by using any of the genotypic methods. With the development of highly sensitive molecular techniques, it is possible to reliably detect even small changes within one species by using PCR or PFGE.

For most of the important diagnostic questions and problems very sensitive methods of molecular differentiation techniques are available today. Also commercially available DNA isolation and purification kits, computer software for compilation and interpretation of the data can easily be put into action [1].

3.1. Evaluation of the methods

New molecular subtyping methods must be evaluated against each other and compared with already established methods. Therefore a set of identical and different well-characterized strains are used to analyze the parameters: typeability, reproducibility and discriminating ability. Typeability means to obtain identical results if the strains are subtyped by the method of choice at the moment and at a later time point. The parameter reproducibility is the percentage of strains classified as the same subtype on repeated testing. It is very difficult to interpret faint bands obtained with universal probes (ribotyping) or artifactual bands; in addition interpretation is complicated by intraspecies variation of restriction patterns [112] or by results caused through incomplete enzyme digestion (as in plasmid or genomic DNA restriction analysis). The use of appropriate controls to ensure reproducibility of the subtyping results cannot be overemphasized [113].

The determination of the discriminating power of a new method – e.g., the ability to differentiate between two unrelated strains – can be evaluated by the use of Simpsons index of diversity [114,115].

This can be defined mathematically as the probability to which unrelated strains will be distinguished by that typing method and may be used to compare typing methods and to select the most discriminatory system.

The implementation of a particular technique for differentiation requires careful consideration of all techniques and a well balanced estimation of the generally known advantages and disadvantages. But one has to keep in mind that the perfect technique – relative to optimal resolution, reproduction and stability – is not yet available.

It is important to establish guidelines for the interpretation of data [68] and also to perform reproducibility studies together with many different laboratories all over the world [71,116]. But it has to be considered that too rigid schemes which arbitrarily define the limits of variations between related bacteria ignore phylogenetic descent and species specific differences in diversity [16]. At the moment, the main problem must be seen in the correct and unbiased interpretation of experimental data.

As long as these uncertainties exist, a combination of at least two typing protocols should be carried out, through which the results of the methods should correspond to each other [1].

The method described by Maiden et al. [9] – the multilocus sequence typing (MLST) – could be the choice of the future, when direct DNA sequencing has been developed into a simple and automated routine DNA analysis technique, allowing different laboratories all over the world to edit their data through a World-Wide-Web.

The comparative quality assessment of the various typing procedures is mandatory, since a large variety of molecular typing methods have been made available. A set of 60 epidemiological well documented *Staphylococcus aureus* strains (epidemiological related and independent) were analysed in a multicenter study [71]. The result of the study showed that in general within genetic procedures a greater resolving power was yielded compared to the phenotypic methods. In a single laboratory all techniques resulted in high reproducibility but a standardised high-quality performance carried out between different laboratories showed low correlation of the results.

This implies that molecular typing methods, though adequate for resolving local problems, may still not be suitable for the standardised analysis of international spread of pathogens when analyzed by several laboratories worldwide. The reasons might be of technical nature, e.g., diversity of PCR machines, Taq-polymerases, electrophoresis supplies etc. Comparing the genotypic methods PFGE and AP-PCR in multicentered studies [117–119], an apparent lack of absolute reproducibility was revealed. For none of

the genetic techniques, reproducibility has been experimentally proven yet in a multicenter study [1].

3.2. Application of subtyping methods

For analysing a nosocomial infection – e.g. infections occurring during hospitalization – it is necessary to differentiate between the isolates of one species and to make a statement about the source of infection and the ways of transmission. The most important question is, if there is a clonal identity of different bacterial isolates from one infection. Such epidemiologic information allows not only statements about possible nosocomial infections but can also answer the question whether various isolates were obtained during different periods or if they represent a new infection or a relapse of a patient. Only with this knowledge, the source of infection and the ways of transmission can be identified and this is the prior condition for effective protection of susceptible patients in the hospital.

For some time, multiresistant strains of the bacterium *Staphylococcus aureus* – particularly the methicillin-resistant strains (MRSAs) – have been causing problems in the struggle of nosocomial infections [120].

Nosocomial infections are a permanent menace for patients. Diverse studies demonstrate that worldwide the rate of nosocomial infections is in the range of 3.5 to 12% [121–125]. In Germany about 3.46% of the patients acquire a nosocomial infection during hospitalization. The annual economic burden of nosocomial infections in the United States is estimated to be more than US$4.5 billion in 1992 [126].

With the new genotypic standardization methods, the analysis of the epidemiology of infectious germs and furthermore clinical hygiene will receive a new dimension. It is much to be hoped that the resulting perceptions will take effect on edict and performance of new regulations to prevent infections.

4. List of abbreviations

AP-PCR	Arbitrary primed-PCR
DNA	Desoxyribonucleic acid
ERIC-PCR	Enterobacterial repetitive intergenic consensus-PCR
ET	Electrophoretic type
HAV	Hepatitis A virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IS	Insertion sequence
LCR	Ligase chain reaction
LRFP	Large restriction fragment pattern
MRSA	Methicillin resistant *Staphylococcus aureus*
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplified polymorphism DNA
REP	Repetitive chromosomal elements
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SSCP	Single-strand conformation polymorphism

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References

[1] A. van Belkum, Med. Microbiol. Lett. 5 (1996) 271.
[2] E.G.M. Power, J. Hosp. Infect. 34 (1996) 247.
[3] S. Weber, M.A. Pfaller, L.A. Herwaldt, Infect. Dis. Clin. North America 11 (1997) 257.
[4] D.A. Relman, J. Infect. Dis. 168 (1993) 1.
[5] K.H. Wilson, Clin. Infect. Dis. 18 (1994) 958.
[6] B. Kreiswirth, J. Kornblum, R.D. Arbeit, W. Eisner, J.N. Maslow, A. McGeer, D.E. Low, R.P. Novick, Science 259 (1993) 227.
[7] J. Hacker, G. Blum-Oehler, I. Mühldorfer, H. Tschäpe, Mol. Microbiol. 23 (1997) 1089.
[8] J.D.A. van Embden, M.D. Cave, J.T. Crawford, J.W. Dale, K.D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T.M. Shinnick, P.M. Small, J. Clin. Microbiol. 31 (1993) 406.
[9] M.C.J. Maiden, J.A. Bygraves, E. Feil, G. Morelli, J.E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D.A. Caugant, M. Achtman, B.G. Spratt, PNAS 95 (1998) 3140.

[10] C.M. Fraser, J.D. Gocayne, O. White, M.D. Adams, R.A. Clayton, R.D. Fleischmann, et al., Science 270 (1995) 397.
[11] R.D. Fleischmann, M.D. Adams, O. White, R.A. Clayton, E.F. Kirkness, A.R. Kerlavage, et al., Science 269 (1995) 496.
[12] R. Himmelreich, H. Hilbert, H. Plagens, E. Pirkl, B.C. Li, R. Hermann, Nucleic Acids Res. 24 (1996) 4420.
[13] J.F. Tomb, O. White, A.R. Kerlavage, R.A. Clayton, G.G. Sutton, R.D. Fleischmann, et al., Nature 388 (1997) 539.
[14] F.R. Blattner, G. Plunkett III, C.A. Bloch, N.T. Perna, V. Burland, M. Riley, J. Collado-Vides, J.D. Glasner, C.K. Rode, G.F. Mayhew, J. Gregor, N.W. Davis, H.A. Kirkpatrick, M.A. Goeden, D.J. Rose, B. Mau, Y. Shao, Science 277 (1997) 1453.
[15] C.M. Fraser, S. Casjens, W.M. Huang, G.G. Sutton, R. Clayton, R. Lathigra, et al., Nature 390 (1997) 580.
[16] M. Achtman, in: M. Sussman (Ed.), Molecular Medical Microbiology, Academic Press, 1998, in press.
[17] K. Wachsmuth, Infect. Control. 6 (1985) 100.
[18] J.J. Mekalanos, J. Bacteriol. 174 (1992) 1.
[19] J.M. Boyce, G. Potter Bynoe, S.M. Opal, L. Dziobek, A.A. Medeiros, J. Infect. Dis. 161 (1990) 493.
[20] P. Beltran, S.A. Plock, N.H. Smith, T.S. Whittam, D.C. Old, R.K. Selander, J. Gen. Microbiol. 137 (1991) 601.
[21] P.L. Meenhorst, A.L. Reingold, D.G. Groothuis, G.W. Gorman, H.W. Wilkinson, R.M. McKinney, J.C. Feeley, D.J. Brenner, R. van Furth, J. Infect. Dis. 152 (1985) 356.
[22] W.R. Grandsen, S.J. Eykyn, I. Phillips, Br. Med. J. 190 (1985) 505.
[23] T.S. Whittam, I.K. Wachsmuth, R.A. Wilson, Infect. Dis. 157 (1988) 1124.
[24] H.L. DuPont, Infect Control Hosp. Epidemiol. 12 (1991) 707.
[25] S. Paton, L. Nicolle, M. Mwongera, P. Kabiru, N. Mirza, F. Plummer, I. Wamola, Infect. Control Hosp. Epidemiol. 12 (1991) 710.
[26] B. Wilske, V. Preac-Mursic, U. Göbel, B. Graf, S. Jauris-Heipke, E. Soutschek, E. Schwab, G. Zumstein, J. Clin. Microbiol. 31 (1993) 340.
[27] K. Kersters, in: M. Goodfellow, D. Jones, F.G. Priest (Eds.), Computer Assistent Bacterial Systematics, Academic Press, London, 1985, p. 337.
[28] P.J.H. Jackman, in: R.R. Colwell, R. Grigorova (Eds.), Methods in Microbiology, Vol. 19, Academic Press, London, 1987, p. 209.
[29] M.E. Mulligan, L.R. Peterson, R.Y.Y. Kwok, C.R. Clabob, D.N. Gerding, J. Clin. Microbiol. 26 (1988) 41.
[30] M.E. Mulligan, R.Y.Y. Kwok, D.M. Citron, J.F. John, P.B. Smith, J. Clin. Microbiol. 26 (1988) 2395.
[31] B. Wilske, V.A.G. Barbour, S. Bergström, N. Burman, B.I. Restrepo, P.A. Rosa, T. Schwan, E. Soutschek, R. Wallich, Res. Microbiol. 143 (1992) 583.
[32] R. Boehmer, G. Will, U. Busch, C. Schweizer, S. Jauris-Heipke, D. Roessler, B. Wilske, V. Preac-Mursic, Med. Microbiol. Lett 4 (1995) 180.
[33] B. Wilske, U. Busch, H. Eiffert, V. Fingerle, H.W. Pfister, D. Rössler, V. Preac-Mursic, Med. Microbiol. Immunol. 184 (1996) 195.
[34] R.K. Selander, D.A. Caugant, H. Ochman, J.M. Musser, M.N. Gilmour, T.S. Whittam, Appl. Environ. Microbiol. 51 (1986) 873.
[35] H. Ochman, R.K. Selander, J. Bacteriol. 157 (1984) 690.
[36] R.K. Selander, J. Li, K. Nelson, in: R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella*, ASM Press, Washington, DC, 1996, p. 2691.
[37] A. Seiler, R. Reinhard, J. Sarkari, D.A. Caugant, M. Achtman, Mol. Microbiol. 19 (1996) 841.
[38] M. Arthur, R.D. Arbeit, C. Kim, P. Beltran, H. Crowe, S. Steinbach, C. Campanelli, R.A. Wilson, R.K. Selander, R. Goldstein, Infect. Immun. 58 (1990) 471.
[39] L.W. Mayer, Clin. Microbiol. Rev. 1 (1988) 228.
[40] A.E. Simor, B. Shames, B. Drumm, P. Sherman, D.E. Low, J.L. Penner, J. Clin. Microbiol. 28 (1990) 83.
[41] A. Hartmann, N. Amarger, Can. J. Microbiol. 37 (1991) 600.
[42] G.L. Archer, A.W. Kachmer, N. Vishniarsky, J.L. Johnson, J. Infect. Dis. 149 (1984) 913.
[43] D.M. Shlaes, C.A. Curie-McCumber, Rev. Infect. Dis. 8 (1986) 738.
[44] F. Marco, M.T. Jimemez de Anta, Enfermedades Infect. Microbiol. Clin. 11 (1993) 97.
[45] F.W. Hyde, R.C. Johnson, J. Clin. Microbiol. 20 (1984) 151.
[46] F.W. Hyde, R.C. Johnson, Zbl. Bakt. Hyg. A. 263 (1986) 119.
[47] R.H.A. Plasterk, M.I. Simon, A.G. Barbour, Nature 318 (1985) 257.
[48] A.G. Barbour, J. Clin. Microbiol. 26 (1988) 475.
[49] A.G. Barbour, Zbl. Bakt. Suppl. 18 (1989) 1.
[50] Y. Xu, R.C. Johnson, J. Clin. Microbiol. 33 (1995) 2679.
[51] U. Busch, C. Hizo-Teufel, R. Boehmer, V. Fingerle, H. Nitschko, B. Wilske, V. Preac-Mursic, J. Clin. Microbiol. 34 (1996) 1072.
[52] E.M. Southern, J. Mol. Biol. 98 (1975) 503.
[53] P.W.M. Hermans, D. van Soolingen, J.W. Dale, A.R.J. Schuitema, R.A. McAdam, D. Catty, J.D.A. van Embden, J. Clin. Microbiol. 28 (1990) 2051.
[54] F.C. Tenover, R.D. Arbeit, R.V. Goering, Infect. Control Hosp. Epidemiol. 18 (1997) 426.
[55] T. Popovic, C.A. Bopp, O. Olsvik, J.A. Kiehlbauch, in: D.H. Pershing, T.F. Smith, F.C. Tenover, T.J. White (Eds.), Diagnostic Molecular Microbiology, ASM Press, Washington, DC, 1993, p. 573.
[56] J.J. LiPuma, M.C. Fisher, S.E. Dasen, J.E. Mortensen, T.L. Stull, J. Infect. Dis. 164 (1991) 133.
[57] M.E. Gordillo, K.V. Singh, B.E. Murray, J. Clin. Microbiol. 31 (1993) 1570.
[58] G. Prevost, B. Jaulhac, Y. Piemont, J. Clin. Microbiol. 30 (1992) 967.
[59] M.A. Pfaller, C. Wendt, R.J. Hollis, P.R. Wenzel, S.J. Fritschel, J.J. Neubauer, L.A. Herwaldt, Diag. Microbiol. Infect. Dis. 25 (1996) 1.
[60] C.A. Gustaferro, in: D.H. Pershing, T.F. Smith, F.C. Tenover, T.J. White (Eds.), Diagnostic Molecular Microbiology, ASM Press, Washington, DC, 1993, p. 584.
[61] M. Altwegg, F.W. Hickman-Brenner, J.J. Farmer III, J. Infect. Dis. 160 (1989) 145.

[62] A. Nastasi, C. Mammina, M.R. Villafrate, Epidemiol. Infect. 107 (1991) 565.
[63] M.W. McDonell, M.N. Simon, F.W. Studier, J. Mol. Biol. 110 (1977) 119.
[64] D.C. Schwartz, C.R. Cantor, Cell 37 (1984) 67.
[65] G. Chu, D. Volrath, R.W. Davis, Science 234 (1986) 1582.
[66] C.R. Cantor, C.L. Smith, M.K. Mathew, Annu. Rev. Biophys. Chem. 17 (1988) 287.
[67] K. Gardiner, Anal. Chem. 63 (1991) 658.
[68] F.C. Tenover, R.D. Arbeit, R.V. Goering, P.A. Mickelsen, B.E. Murray, D.H. Pershing, J. Clin. Microbiol. 33 (1995) 223.
[69] A.C. Whelen, D.H. Pershing, Annu. Rev. Microbiol. 50 (1996) 349.
[70] R.V. Goering, Infect. Control Hosp. Epidemiol. 14 (1993) 595.
[71] F. Tenover, R.D. Arbeit, G. Archer, J. Biddle, S. Byrne, R. Goering, G. Hancock, G.A. Hebert, B. Hill, R. Hollis, W.R. Jarvis, B. Kreiswirth, W. Eisner, J. Maslow, L.K. McDougal, J.M. Miller, M.E. Mulligan, M.A. Pfaller, J. Clin. Microbiol. 32 (1994) 407.
[72] U. Busch, C. Hizo-Teufel, R. Boehmer, B. Wilske, V. Preac-Mursic, Electrophoresis 16 (1995) 744.
[73] U. Busch, C. Hizo-Teufel, R. Boehmer, V. Preac-Mursic, Ann. Acad. Med. Bialostocensis 41 (1996) 51.
[74] U. Busch, C. Hizo-Teufel, R. Boehmer, V. Fingerle, D. Rössler, B. Wilske, V. Preac-Mursic, Scand. J. Infect. Dis. 28 (1996) 583.
[75] U. Busch, G. Will, C. Hizo-Teufel, B. Wilske, V. Preac-Mursic, Res. Microbiol. 148 (1997) 109.
[76] T.M.A. Weller, F.M. MacKenzie, K.J. Forbes, J. Med. Microbiol. 46 (1997) 921.
[77] M. Cruciani, M. Malena, G. Amalfitano, P. Monti, L. Bonomi, Clin. Infect. Dis. 26 (1998) 330.
[78] A.E. Fiore, J. Pekka Nuorti, O.S. Levine, A. Marx, A.C. Weltman, S. Yeager, R.F. Benson, J. Pruckler, P.H. Edelstein, P. Greer, S.R. Zaki, B.S. Fields, J.C. Butler, Clin. Infect. Dis. 26 (1998) 426.
[79] J.M. Martin, E.R. Wald, M. Green, J. Infect. Dis. 177 (1998) 504.
[80] J. Belfaiza, D. Postic, E. Bellenger, G. Baranton, I. Saint-Girons, J. Clin. Microbiol. 31 (1993) 2873.
[81] R.T. Marconi, C.F. Garon, J. Clin. Microbiol. 30 (1992) 2830.
[82] V. Gürtler, V. Stanisich, Microbiology 142 (1996) 3.
[83] M. McClelland, C. Petersen, J. Welsh, J. Clin. Microbiol. 30 (1992) 1499.
[84] O.G. Brakstad, J.A. Maeland, Y. Tveten, APMIS 101 (1993) 681.
[85] T.A. Cebula, W.L. Payne, P. Feng, J. Clin. Microbiol. 33 (1995) 248.
[86] F.-J. Schmitz, C.R. MacKenzie, B. Hofmann, J. Verhoef, M. Finken-Eigen, H.-P. Heinz, K. Köhrer, J. Med. Microbiol. 46 (1997) 773.
[87] M.N. Widjojoatmodjo, A.C. Fluit, J. Verhoef, J. Clin. Microbiol. 32 (1994) 3002.
[88] C.A. Gaydos, L. Bodo, L. Welsh, E.W. Hook III, Sex. Trans. Dis. 19 (1992) 303.
[89] E. Navarrow, P. Simonet, P. Normand, R. Bardin, Arch. Microbiol. 157 (1992) 107.
[90] C. Garzelli, N. Lari, B. Nguon, M. Cavallini, M. Pistello, G. Falcone, J. Med. Microbiol. 46 (1997) 933.
[91] C.S. Hulton, C.F. Higgins, P.M. Sharp, Mol. Microbiol. 5 (1991) 825.
[92] J. Versalovic, K. Thearith, J.R. Lupski, Nucleic Acids Res. 19 (1991) 6823.
[93] C. Breukel, J. Wijnen, C. Tops, H. van der Klift, H. Dauwerse, P.M. Khan, Nucleic Acids Res. 18 (1990) 3097.
[94] J. Welsh, M. McCelland, Nucleic Acids Res. 18 (1990) 7213.
[95] J.G.K. Williams, A.R. Kubelik, K.J. Livak, J.A. Rafalski, S.V. Tingey, Nucleic Acids Res. 18 (1990) 6531.
[96] N. Akopyanz, N.O. Bukanov, T.U. Westblom, S. Kresovich, D.E. Berg, Nucleic Acids Res. 20 (1992) 5137.
[97] S.I. Makino, Y. Okada, T. Maruyama, S. Kaneko, C. Sasakawa, J. Clin. Microbiol. 32 (1994) 65.
[98] C.J. Linton, H. Jalal, J.P. Leeming, M.R. Millar, J. Clin. Microbiol. 32 (1994) 2169.
[99] A. Coelho, A.C.P. Vicente, M.A.S. Baptista, H. Momen, F.A.R. Santos, C.A. Salles, Res. Microbiol. 146 (1995) 671.
[100] E. Mahenthiralingam, M.E. Campbell, J. Foster, J.S. Lam, D.P. Speert, J. Clin. Microbiol. 34 (1995) 1129.
[101] E. Mahenthiralingam, M.E. Campbell, D.A. Henry, D.P. Speert, J. Clin. Microbiol. 34 (1995) 2914.
[102] A.W. Lin, M.A. Usera, T.J. Barrett, R.A. Goldsby, J. Clin. Microbiol. 34 (1996) 870.
[103] J.P. Woods, D. Kersulyte, R.W. Tolan Jr., C.M. Berg, D.E. Berg, J. Infect. Dis. 169 (1994) 1384.
[104] B.G. Corney, J. Colley, G.C. Graham, J. Med. Microbiol. 46 (1997) 927.
[105] A. Hejazi, C.T. Keane, F.R. Falkiner, J. Med. Microbiol. 46 (1997) 913.
[106] F. Barany, Proc. Natl. Acad. Sci., USA 88 (1991) 189.
[107] R. Weiss, Science 254 (1991) 1292.
[108] P. Fang, S. Bouma, C. Jou, J. Gordon, A.L. Beaudet, Hum. Mutat. 6 (1995) 144.
[109] T.G. Laffler, J.J. Carrion, R.M. Marshall, Ann. Biol. Clin. 50 (1993) 821.
[110] E.S. Winn-Deen, C.A. Batt, M. Wiedmann, Appl. Environ. Microbiol. 60 (1993) 278.
[111] J.S. Lewis, in: S.A. Morse, A.A. Moreland, K.K. Holmes (Eds.), Atlas of Sexually Transmitted Diseases and AIDS, Mosby-Wolfe, London, 1996, p. 319.
[112] M.J. Struelens, Y. De Gheldre, N. Maes, A. Deplano, Med. Microbiol. Lett. 5 (1996) 381.
[113] B. Swaminathan, G.M. Matar, in: D.H. Persing, T.F. Smith, F.C. Tenover, T.J. White (Eds.), Diagnostic Molecular Microbiology, American Society for Microbiology, Washington, DC, 1993, p. 26.
[114] P.R. Hunter, J. Clin. Microbiol. 28 (1990) 1903.
[115] P.R. Hunter, M.A. Gaston, J. Clin. Microbiol. 26 (1988) 2465.
[116] European Study Group on Epidemiological Markers (ESGEM)M.J. Struelens, P. Gerner-Smidt, V. Rosdahl, N. El Sohl, J. Etienne, M.H. Nicolas, U. Romling, W. Witte, N. Legakis, A. van Belkum, L. Dijkshooren, H. de Lencastr, J. Garaizar, D. Blanc, A. Bauernfeind, B.D. Cookson, T.L. Pitt, Clin. Microbiol. Infect. 2 (1996) 2.

[117] A.H. Brandenburg, A. van Belkum, C. van Pelt, H.A. Bruining, J.W. Mouton, H.A. Verbrugh, J. Clin. Microbiol. 34 (1996) 2089.
[118] A. van Belkum, J. Kluytmans, W. van Leeuwen, R. Bax, W. Quint, E. Peters, A. Fluit, C. Vandenbroucke-Grauls, A. van den Brule, H. Koeleman, W. Melchers, J. Meis, A. Elaichouni, M. Vaneechoutte, F. Moonens, N. Maes, M. Strulens, T. Tenover, H.A. Verbrugh, J. Clin. Microbiol. 33 (1995) 1537.
[119] A. van Belkum, Clin. Microbiol. Rev. 7 (1994) 174.
[120] W. Witte, C. Cuny, C. Braulke, D. Heuck, I. Klare, Eurosurveillance 2 (1997) 25.
[121] NIDEP-Study (Nosocomial infections in Germany) Part 1: Schriftenreihe des Bundesministeriums für Gesundheit, Band 56, 1995.
[122] E. Humphreys, A.M. Emmerson, J. Hosp. Infect. 25 (1993) 75.
[123] R.W. Haley, D.H. Culver, J.W. White, W.M. Morgan, T.G. Emori, Am. J. Epidemiol. 121 (1985) 159.
[124] P.D. Meers, G.A.J. Ayliffe, A.M. Emmerson, D.A. Leigh, R.T. Mayon-White, C.A. Mackintosh, J.L. Stronge, J. Hosp. Infect. 2(Suppl.) (1981) 1.
[125] O.B. Jepsen, N. Mortensen, J. Hosp. Infect. 1 (1980) 237.
[126] W.J. Martone, W.R. Jaris, D.H. Culver, R.W. Haley, in: J.V. Bennet, P.S. Brachman (Eds.), Hospital Infections, Little, Brown, Boston, MA, 3rd ed., 1992, p. 577.
[127] H. Schätzl, H.R. Gelderblom, H. Nitschko, K. von der Helm, Arch. Virol. 120 (1991) 71.
[128] G. Frösner (Ed.), Moderne Hepatitisdiagnostik, Kilian Verlag, Marburg, 1996.
[129] H. Nitschko, T. Schwarz, G. Jäger, Pädiatrische Grenzgebiete 34 (1996) 331.
[130] P. Simmonds, E.C. Holmes, T.-A. Cha, J. Gen. Virol. 74 (1993) 2391.
[131] T. Suzuki, E. Tanaka, A. Matsumoto, J. Med. Virol. 46 (1995) 162.
[132] H. Yatsuhashi, S. Han, K. Inokuchi, in: Abstract Book International Symposium on Viral Hepatitis and Liver Disease, 10–14 May 1993, Tokyo, Abstr. No. 625, p. 241.
[133] L. Chemello, A. Alberti, K. Rose, New Engl. J. Med. 330 (1994) 143.
[134] H. Okamoto, S. Mishiro, Intervirology 37 (1994) 68.