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Review Article

Molecular Diagnostics in Clinical Microbiology

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INTRODUCTION

In the daily routine of a clinical microbiological laboratory, pathogens can be detected in several ways. Diagnostics of infectious diseases require a strategic approach; since the etiological agent can be of bacterial, viral, fungal or protozoan origin, frequently sharing an identical syndrome. A complicating factor is that the clinical sample, sent to the laboratory, can be severely contaminated with commensal flora. Moreover, the transport of patient sample to the laboratory and the sample itself can significantly influence the viability of the pathogen (e.g. anaerobes or viruses) and consequently the outcome of the culture. Strict logistic agreements are of fundamental importance. It has been demonstrated that experience and assessment of clinical parameters by the treating medical practitioner will determine the choice of the clinical microbiological procedure. This does not always hold true. Significantly, microbial diagnostics of clinical samples will be performed by microscopy and culture techniques. An additional issue is the non-cultureable and fastidious micro-organisms. Indirect detection of the causative agent such as serology would be possible solutions. This approach may demonstrate pathogen-specific antibodies in the patient's serum. However, a convalescent serum sample (taken two weeks after the 1st sample in the acute phase of the infection) is needed in order to obtain a reliable result. Conventional microbiology is an inexpensive but protracted diagnostic method. Interpretation of the culture results requires technical skill. Rapid

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diagnosis of pathogens (within the same day), needed for cohort screening of humans possibly colonized or infected with a multi-resistant micro-organism, is beyond the power of conventional microbiological approaches.

In the last two decades, strategies based on nucleic acid amplification techniques (NAATs) have taken an irreversible position in the diagnostic field of infectious diseases. Pathogens can be detected in qualitative and quantitative NAAT strategies by selection of species-specific nucleic acid targets. Moreover, NAATs allow a better understanding of mixed population dynamics of both aerobic as well as anaerobic bacteria. The course of an infection, as a consequence of an antimicrobial therapy in combination with the host immune response, can be measured with quantitative diagnostic NA approaches. A number of currently developed molecular-based techniques, such as whole genome sequencing, may play an important role in the development of new screening strategies for direct detection of pathogens in clinical samples.

Detection and identification of the causative infectious agent is a highly relevant issue in microbiological diagnostics. Alternatively, most of the pathogens may be transmitted among humans quite easily, and therefore it is also essential to identify these pathogens below the species level (bacterial typing) to determine its spread among individuals in the hospital environment as well as in the community. Conventional typing methods determine the phenotype of pathogens to assess epidemiological relatedness by analyzing the biochemical or antimicrobial resistance patterns, the sensitivity to lytic bacteriophages (phagetyping) or specific immune reaction to cell wall components (serotyping). These techniques have been used for decades quite successfully; but lack performance (poor resolution and reproducibility or typeability). Currently, genetic typing methods have provided the microbiological diagnostic laboratory with a powerful tool to improve identification on the strain level. This minireview describes the success of novel nucleic acid-based techniques implemented in both pathogen diagnostics and subspecies identification in microbiology.

Molecular diagnostics workflow of pathogen detection and identification in clinical samples. The workflow of molecular diagnostics in the microbiological laboratory is simple and straight forward. Nucleic acid from the potential pathogen is extracted from the clinical sample, subsequently followed by an amplification-detection protocol, preferably in real-time format, in a single or multiplex assay. However, this simple workflow is punctuated with a number of issues. Many effective solutions to avoid these issues have been introduced. Complicated extraction protocols using undesired chemicals are replaced with commercial filter column or magnetic bead-based extraction robots. These systems allow less hands-on-time, high-throughput, nucleic acid isolation and purification from hundreds of clinical samples per working day. Despite this high level of automation, many complex clinical samples such as blood, faeces, tissue, sputa, etc., still require so-called off-board pre-extraction and lysis protocols, which will slow down the process.

An internal nucleic acid control should be introduced to monitor the extraction procedure. This process control identifies the effect of amplification inhibitory compounds from the clinical sample, such as urea or haemoglobin, and loss of sample during extraction. Both phenomena lead to reliable positive and negative sample results. The next logical step in the process is the amplification of the target nucleic acid. The Polymerase Chain Reaction (PCR) is the most frequently used methodology of the numerous amplification techniques that are currently available. Most of the "alternative" amplification reactions, such as Nucleic Acid Sequence-Based Amplification (NASBA), Ligase Chain Reaction (LCR), Transcription-mediated Amplification (TMA) and Strand Displacement Amplification (SDA), are adapted as commercial assays. Amplification reactions always require logistic adaptation of the microbiological laboratory and molecularly skilled technician and staff. The molecular diagnostic procedure, including nucleic acid extraction, amplification and analysis, requires physically separated laboratories, principally to avoid carry-over contamination of amplification products.

The first generation amplification techniques, which are primarily PCR-based, necessitate a post-amplification step. Herewith, the PCR product is detected and identified with a combination of agarose gel electrophoresis and blotting. This qualitative approach has a short dynamic range, a low resolution and moderate sensitivity and is a non-automated and time-consuming procedure. Technical and chemical developments realized a quantitative

and rapid amplification procedure, the real-time PCR. Many different real-time PCR platforms were developed and can be primarily distinguished on sample throughput and sample heating. The PCR products (amplicons) generated during the process can be monitored real-time using (probe-associated) fluorescence detection. Real-time PCR provides a computer-based analysis of the fluorescent time course, an ultra rapid cycle programme (30 min-2 h), a wide dynamic range (10^{10}-fold) and quantitative results. Moreover, this platform uses a closed sample system, which virtually excludes contamination.

The quality of the real-time PCR results can be warranted by laboratory quality control and quality assurance procedures. Quality control refers to a system of process controls, which provides data on the integrity and correctness of the procedure. Quality assurance involves a system of review procedures, performed by an independent institute, such as the QCMD (Quality Control of Molecular Diagnostics). These institutes monitor the correct functioning of quality control systems running on the molecular microbiology diagnostic laboratory. QCMD, founded by the EU, provides external quality assessment programmes for a wide variety of bacterial, parasitological and viral targets. For more information about QCMD's core aims, programmes, etc. see: http://www.qcmd.org.

While the high sensitivity and specificity of amplification techniques is usually extremely useful in the detection of minute amounts of specific microorganisms, these properties can also have disadvantages. Due to the specificity of the amplification methods, they are unable to catch all pathogens in the clinical sample simultaneously, unlike microscopy or culture methods. Multiplex approaches can solve this issue. A second limitation is the sensitivity of the test, amplifying even single copies of a target. High sensitivity is useless when commensal flora is involved. For instance, the mere detection of certain bacteria in samples from the upper respiratory tract hardly has any clinical relevance, since some micro-organisms can both colonize and infect this anatomical niche. In this case, quantification and determination of a clinically relevant threshold for detection are necessary. The detection of a target gene or variations within a gene does not represent the properties of an organism. The phenotype of a living cell is reflected by the interaction and regulation of a number of genes. For instance, conventional culture techniques are still needed for the analysis of an antimicrobial susceptibility pattern of bacteria. Molecular systems have increased the diagnostic power in infectious diseases in general.

Detection and identification of methicillin-resistant *Staphylococcus aureus*, an example. *Staphylococcus aureus* infections in the hospital and in the community impose significant morbidity, mortality and healthcare costs. Usage of antibiotics to eradicate this pathogen frequently leads to the emergence of additional antibiotic resistance traits. Rapid worldwide spread of meticillin-resistant *S. aureus* (MRSA) clones currently results in a multitude of hospital outbreaks, although implementation of strict infection control measurements in some countries has kept the MRSA prevalence low. Effective infection prevention to restrict dissemination of MRSA depends on the reliability and speed of antibiotic resistance detection by the microbiology laboratory. This emphasizes the clinical and epidemiological need for high speed detection, preferably directly from clinical specimens. Rapid molecular diagnostic methods target resistance genes and have proven to be excellent and robust tools to either confirm the clinically relevant MRSA phenotype and detect MRSA colonisation and/or infection direct from clinical specimens within a single work day.

Conventional *S. aureus* detection and identification. Firstly, *S. aureus* has to be distinguished from other staphylococcal species. Based on the detection of surface components by, for instance, latex agglutination assays, *S. aureus* can be identified to the species level (1). False - positive results through cross-reactivity with other staphylococcal species may occur occasionally. The current gold standard method to identify *S. aureus* from cultures is the AccuProbe *Staphylococcus aureus* Culture Identification Test (Gen-Probe). It has to be stated, however, that many diagnostic laboratories still rely on colony colour and morphology assessment in combination with latex agglutination testing for diagnosing *S. aureus*.

Screening for antibiotic resistance determinants in *S. aureus*. The main hospital-based reservoirs of MRSA are the colonized and/ or infected patients, the colonized healthcare workers and the environment. Early recognition of patients colonized or infected with

MRSA should have a direct impact on the selection of antimicrobial therapy and should facilitate decisions to initiate infection prevention measures. In countries with low MRSA endemicity, at risk patients are isolated until the MRSA diagnostic test has confirmed the absence of MRSA. Culture-based techniques will take 3-5 days leading to unnecessary lengthy isolation for the vast majority of possibly nasal *S. aureus* colonized patients. "Aggressive" selective enrichment, introduced for optimal performance of the test, is the main reason for this delay (2). There is a clear need for rapid detection and identification of bacteria directly from patient samples. Rapid methods based on immunological or molecular technologies or combinations thereof have contributed significantly to the speed, reliability, sensitivity and specificity of MRSA testing. Below, the molecular targets used for MRSA detection will be defined and the test systems that are currently available will be described.

Clinically relevant meticillin resistance in *S. aureus* is the result of the acquisition of an alternative penicillin binding protein (PBP2a) encoded by the *mecA* gene, which has a low affinity for most of the beta-lactam antibiotics (3). The *mecA* gene is carried on a mobile genetic element, SCC*mec* (Staphylococcal Cassette Chromosome *mec,* see Fig. 1).

Integration of the SCC*mec* into the staphylococcal chromosome takes place at a conserved attachment site (*orfX*) near the origin of DNA replication. The ability of *S. aureus* to accommodate SCC*mec* and/or to functionally integrate PBP2a differs from strain to strain, resulting in a wide range of resistance levels.

Molecular screening methods for MRSA detection and identification. Conventional culture methods still remain the predominant approach for detection and identification of MRSA. A major problem in classical MRSA diagnosis is the variable phenotypic expression of the *mecA* gene-dependent methicillin resistance. Strains with a heterogeneous resistance may result in false-negative outcomes and form a challenge to the laboratory. Several immunological latex agglutination tests have been developed to detect the product of the *mecA* gene. The principle of the latex agglutination (LA) test depends on the presence of PBP2a (4). The latex particles are coated with anti-PBP2a monoclonal antibodies and will agglutinate with a suspension of a MRSA colony. A disadvantage of this immulogical approach is the influence of the *mecA* gene expression level. Inducible isolates, i.e. isolates that harbour the *mecA* gene and a complete set of regulatory genes, have minimal or no *mecA* expression, giving weak or no agglutination reaction or agglutinate slowly (5).

PCR, based on the detection of the methicillin resistance determinant *mecA*, is still considered to be the gold standard molecular-diagnostic tool for MRSA. PCR assays which detect a single target (*mecA*) are both robust and easy to perform (6). However, amplification inhibition may lead to false-negative results. Addition of a second target sequence, present in all *S. aureus* strains, can solve this problem. "Internal control" markers were applied to identify *S. aureus*, such as *nuc* (7), a thermostable nuclease, *gyrA* (8), or a 442 bp-fragment named *holB* (*SA442*) present in all *S. aureus* isolates tested (9), 16S ribosomal RNA gene (10,11), *femA* (12,13), *femB* (14). The latter two genes are involved in the peptidoglycan synthesis of *S. aureus.* One should be aware, however, that gene polymorphism, such as primer annealing site polymorphism may occur and MRSA strains can be misidentified (15,16). The above mentioned methods are generally applicable for the identification of MRSA from purified "suspicious" cultures. Direct MRSA detection from the clinical sample, however, is the ultimate goal.

A major obstacle in direct MRSA detection from clinical samples is co-colonization with clinically insignificant meticillin-resistant, coagulase-negative staphylococci (MRCoNS), which also carry the *mecA* gene. The presence of these bacteria in clinical samples may result in false-positive outcomes when only the *mecA* gene is used as PCR target (17,18). High rates of MRCoNS have been reported for clinical centers in central Europe and other regions, ranging from 70 to 80% (18,19). Diverse approaches have been developed to increase the specificity. These include a selective enrichment broth prior to amplification. A strategy has been developed to counter the problem of clinical samples confounded by the presence of *mecA*-positive CoNS. This approach is based on PCR amplification of an *S. aureus*-specific chromosomal DNA fragment (*orfX*) adjacent to SCC*mec* and a fragment within SCC*mec* (20-22). This PCR assay has been converted in a commercially available test system, i.e. the *BD GeneOhm MRSA*™ kit (Becton Dickinson, Alphen aan de Rijn, The Netherlands). The performance of the test was evaluated with 1657

MRSA and 569 MSSA strains and was reported to correctly identify 98.7% of the strains, whereas 4.6% of the meticillin-susceptible strains were misidentified (22).

The evaluation of direct MRSA screening from nasal swabs was established and compared to conventional culture methods (23). The diagnostic values were 91.7% for sensitivity, 93.5% for specificity, 82.5% for the positive predictive value, and 97.1% for the negative predictive value when compared to culture-based methods. Six false-negative results were obtained. Four strains were retested and three were found to be *mecA*-negative. An explanation for the failure of the assay to detect

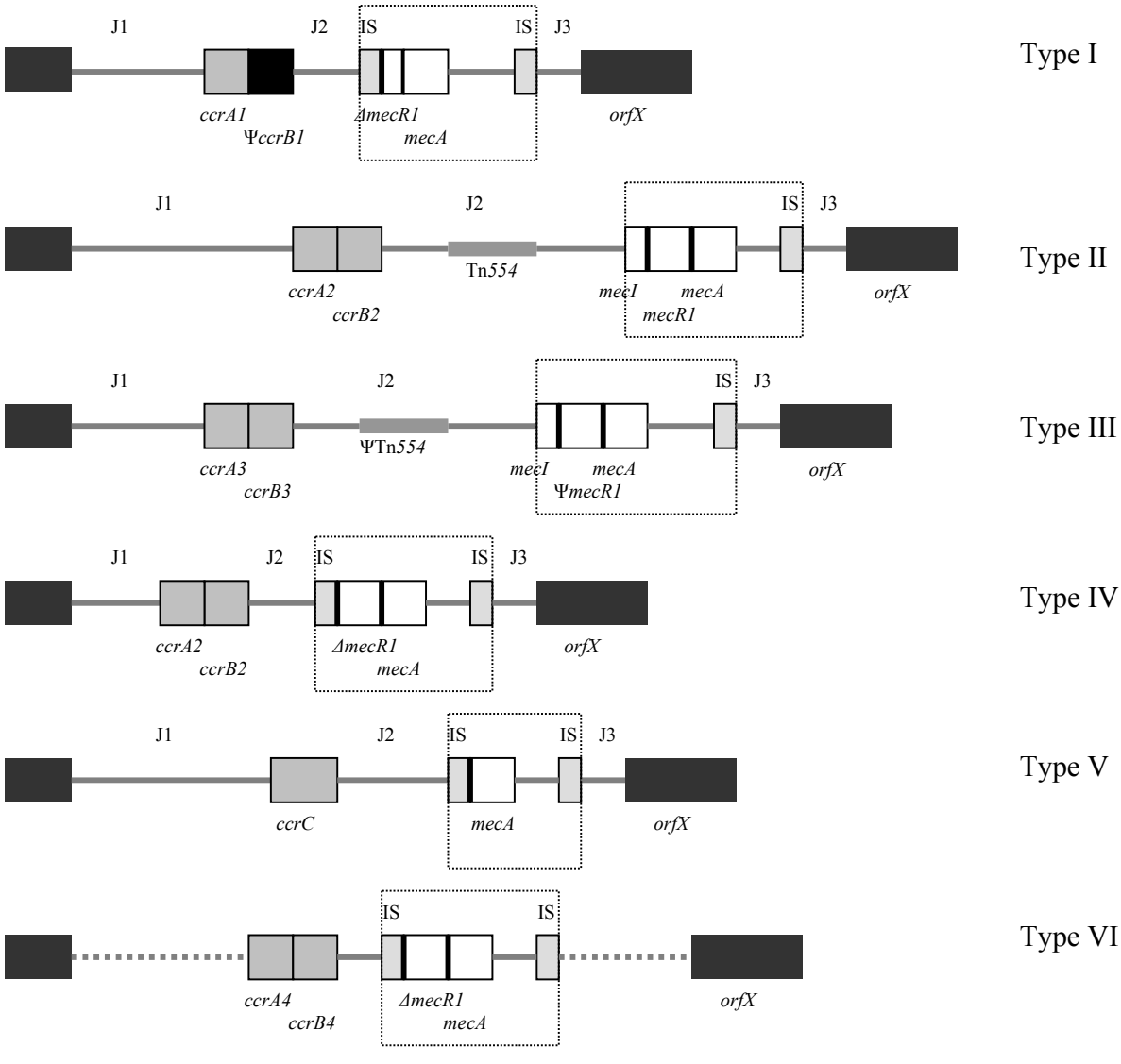

Fig. 1. Organization of the known SCCmec types. SCCmec elements share four characteristics: (1) the mec gene complex (dotted boxes) consisting of mecA, the meticillin resistance determinant, presence or absence of (parts of) its regulatory genes and insertion sequences (IS); (2) presence of the cassette chromosome recombinase (ccr) genes responsible for the mobility of the SCCmec element; (3) presence of direct- and inverted complementary repeat sequences at both ends of the element; (4) integration of the element on the staphylococcal chromosome into the 3'-end of open reading frame X (*orfX*). SCC*mec* type definition is based on the identification of its components: *ccr* genes (5 types), *mec* complex (4 classes) and specific structures in junkyard (J) regions (plasmids and transposons). The subtypes (not indicated) within the SCC*mec* types II and III are characterized by junkyard sequence variability.

MRSA is either the limitation of the assay regarding sensitivity or the emergence of previously unknown SSC*mec* sequences.

Multiple new molecular technologies using e.g. recombinase polymerase amplification (RPA) (24), or nucleic acid sequence based amplification (NASBA) assays seem to have an increased sensitivity and specificity for detecting MRSA. However, those tests target the highly variable SCC*mec-orfX* region and for this reason, a continuous renewal and optimization of the test is needed.

New strategies for the identification of micro-organisms, which are not based on nucleic acid amplification, are spectroscopy-based methods, such as Raman and mass spectroscopy. These technologies analyse the complete biochemical composition of micro-organisms and can provide a species-specific fingerprint (25,26). Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) has been used to discriminate between MRSA and MSSA strains (27). However, the preliminary results showed lack of reproducibility (media effect on spectra), sensitivity (culture is inevitable) and, hence, speed (27,28).

In brief, the clinical microbiology laboratory is slowly turning its back on the technologies developed in the ages of Pasteur and Koch. Molecular technology has changed the horizon and for *Chlamydia trachomatis* detection it already is the gold standard technology. That molecular testing will also revolutionize MRSA detection is obvious. It remains to be seen which of the many currently available technologies will in the end be collectively embraced by the majority of clinical microbiologists.

Bacterial genome comparison. Pathogenic bacteria reside in several reservoirs, such as humans , animals, food, and water. Dissemination of these bacteria from any of the ecological niches may set up clusters of colonization or infections among humans. When these clusters, recognized as outbreaks, are not controlled, further transmission will occur, which may subsequently lead to a pandemic. Bacteria can be classified on the strain level with epidemiological typing systems, which identify isolate-specific characters, the so-called epidemiological markers. The products of typing methods: fingerprints, sequence types, spectroscopic results, or micro-array patterns can be compared with each other and can be used to elucidate the source and transmission routes of pathogenic bacteria.

Purpose of epidemiological typing. Typing methods can be used to determine the spread of micro-organisms among individuals in healthcare or environmental settings. In other words, these methods are used for epidemiological studies, such as for instance infectious disease outbreak investigation, aim to define genetic relationships among strains which are isolated from individuals hospitalized or working within a restricted area (hospital ward) and within a short period of time (days, weeks). Other studies, e.g. long-term epidemiological surveillance of infectious diseases or the analysis of the population structure analysis or taxonomy, address the relationship between strains recovered during extended periods of time (years, decades) and over a broader geographical level (nation-, worldwide).

Bacterial typing is most frequently used for outbreak investigation. An outbreak is defined as a local and temporal increase in the frequency of colonization and/or infection by a given microorganism. For example, hospital infection control is alerted in the case of a conspicuous increase in the rate of isolation of a specific pathogen, possibly exhibiting an unusual antibiogram, or a cluster of infections in a hospital ward. In these situations, answers to questions of strain relatedness may be elucidated by typing data (29-31). Comparative typing is applied to facilitate the development of outbreak control strategies, and address questions regarding the extent of epidemic spread of microbial clones, the number of clones involved in transmission and infection, the monitoring of reservoirs of epidemic clones or for the evaluation of the efficacy of control measures.

Criteria for the evaluation of typing systems. Several parameters should be considered when evaluating typing systems (32-35). The performance criteria include the typeability, reproducibility, stability, and discriminatory power of a typing system. Typeability refers to the ability of a system to obtain a positive result for each isolate analyzed and is influenced by both technical and biological factors. The technical reproducibility is the ability to assign the same type to a strain tested on independent occasions. The biological reproducibility or stability of epidemiological markers is the ability of a typing system to recognize clonal relatedness of strains derived from a common ancestor. Phenotypic or genomic variation may occur during storage or replication of strains in the laboratory (in-vitro stability). Clonal expansion of a strain over a long

period of time or during geographically wide-spread outbreaks (in-vivo stability) can also result in various degrees of genetic variation. The discriminatory power refers to the average probability that a typing system will assign different types to two unrelated strains. Ideally, each unrelated strain is identified as unique (36,37). Considering the performance criteria, the epidemiological question determines the choice of the applied typing technique.

Classification of typing methods. A convenient basis for classifying typing systems is to recognize them as phenotypic techniques, those that detect characteristics expressed by microorganisms, and genotypic techniques, those that involve direct nucleic acid-based analysis of chromosomal or extra-chromosomal genetic elements.

Historically, the identification and characterization of bacterial isolates has been achieved by phenotypic analyses and for many decades, have served as the basis for epidemiological analyses. Phenotypic methods are those that characterize products of gene expression in order to identify the species level or to differentiate strains. Properties such as biochemical profiles, susceptibility to bacteriophages, antigens present on the cell's surface, whole protein analysis (38-40) and antimicrobial susceptibility patterns were used as epidemiological targets. All are examples of phenotypic properties that can be determined in the microbiology laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on environmental influences. For this reason, phenotyping assays are often limited in reproducibility or reliability. Moreover, these systems lack typeability, discriminatory power and, consequently, are not the most adequate approaches for bacterial comparison.

The advances of molecular biology have resulted in the development of multiple DNA-based strain typing

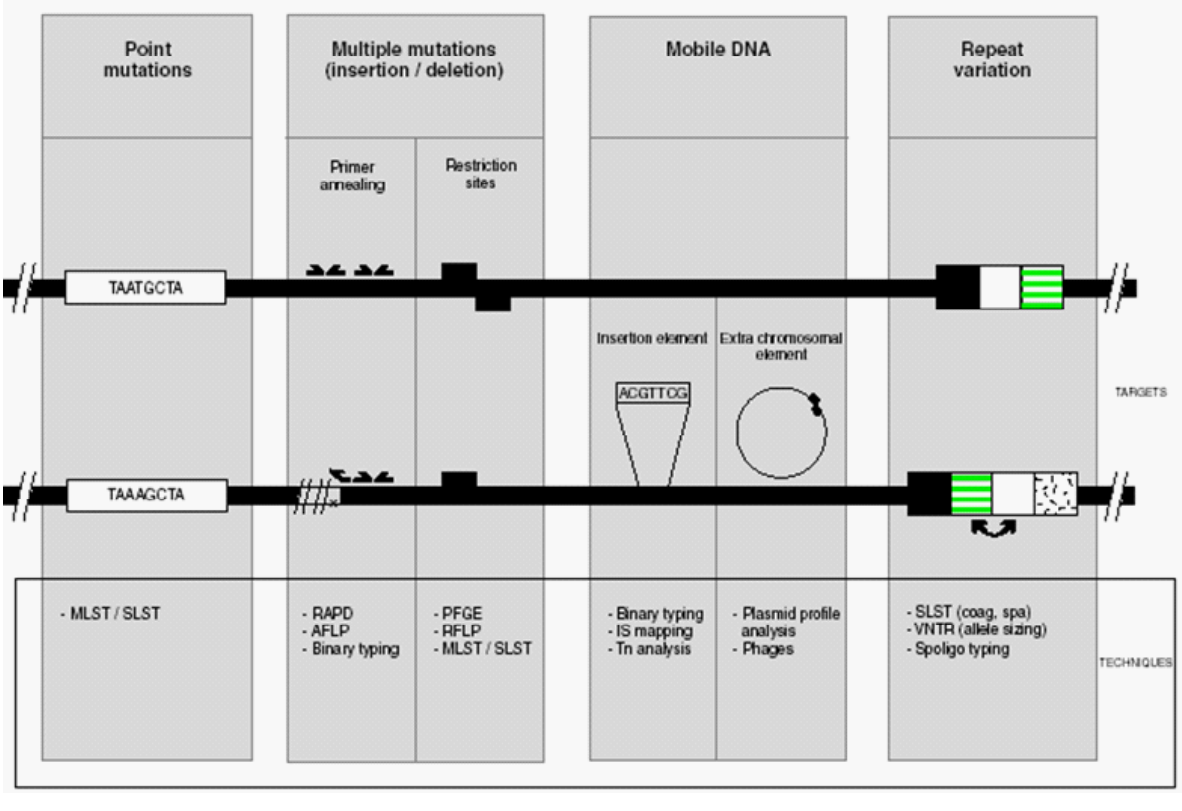

Fig. 2. The molecular basis for the comparison of bacterial genomic DNA molecules: targets and techniques. The currently developed genotyping approaches for the discrimination of bacterial strains measure variability in single nucleotides, insertion or deletion of DNA fragments, presence or absence of (extra) chromosomal mobile DNA elements (plasmids, transposons, insertion sequences, phages, pathogenicity- and resistance islands), and polymorphisms in the frequency of DNA repeat sequences. The different strategies to detect the different targets are: MLST, multi-locus sequence typing; SLST, single-locus sequence typing; RAPD, randomly amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism; IS, insertion sequence; Tn, transposon; VNTR, variable number of tandem repeats.

strategies. The molecular basis of the different techniques for discriminating individual DNA molecules and the respective targets are summarized in Fig. 2.

Over the last two decades DNA-based technologies have been introduced and are increasingly being used in clinical laboratories, which are reflected by the number of papers reporting on bacterial epidemiology (41). Over time, several stages of molecular typing methods have found their application in the analysis of bacterial strain collections (42). These laboratory developments are reviewed chronologically here.

First-phase molecular typing: plasmid profile analysis. The first DNA-based techniques applied to epidemiological studies involved the analysis of plasmids, which were introduced in the mid-1970s (43-45). Bacterial plasmids are autonomously replicating extra-chromosomal elements, distinct from the chromosome. The analysis of plasmids is a technically simple process. However, many isolates of different bacterial species lack them and can, therefore, not be typed by this approach (46-49). Also, the reproducibility of plasmid profiling is confounded by structure variability of the plasmid itself (supercoiled, nicked, linear and oligomeric). This problem can be circumvented by the digestion of the plasmids into restriction fragments and analyzing their numbers and sizes. The fundamental drawbacks have limited the application of plasmid analysis and the method has only proven effective for evaluating isolates under restricted temporal and geographical conditions such as during an acute outbreak episode in a single hospital.

Second-phase molecular typing: Southern hybridization analysis of digested chromosomal DNA. The bacterial chromosome is the prime target molecule for the measurement of relationship between bacterial cells. Classical Southern blot analysis detects only specific restriction fragments carrying DNA sequences homologous to the probe used (50). The choice of the probe is a critical consideration with respect to typeability and discriminatory power and is directly related to the frequency with which the detected restriction fragments vary in number, size, or both (Fig. 3). The best-known hybridization-mediated typing procedure is ribotyping. DNA probes corresponding to (parts of) ribosomal genes are used to highlight polymorphisms (51-54). The complete ribotyping procedure has recently been automated and

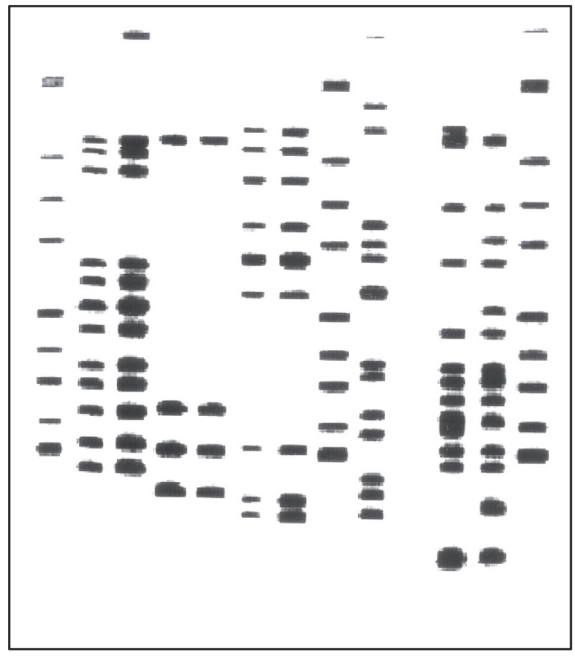

Fig. 3. Southern hybridization analysis. Genomic DNA of 13 MRSA strains was digested with a frequently cutting restriction enzyme. Restriction fragments were separated by size through agarose gel electrophoresis and subsequently transferred onto a nylon membrane. The immobilized DNA restriction fragments are hybridized with a radioactively-labeled 16S-specific probe and detected by autoradiography.

in the case of MRSA the results have been coupled to a database management system (55). This library system should facilitate inter- center data exchange, which is explored by ongoing multicenter studies, such as GENE (Genetic Epidemiology Network for Europe, S. Brisse, Utrecht, the Netherlands; Qualicon Riboprinter as core method), an EU sponsored concerted action.

Third-phase molecular typing: PCR-based techniques and puls-field gel electrophoresis.

Restriction digestion of PCR products. The PCR products (amplicons) can be digested with specific DNA-restriction endonuclease(s). The DNA fragment length between the restriction sites can be variable. These restriction fragment length polymorphisms (RFLPs) can be analyzed by gel electrophoresis. The discrimination of strains with this technique is moderate (56,57). The resolution can be improved by increasing the number of loci analyzed, or by increasing the number of restriction enzymes per locus analyzed (58).

PCR based on repetitive chromosomal sequences. Short extragenic repetitive sequences, originally identified in *Enterobacteriaceae* can be used as templates for PCR (59,60). Repetitive interspersed sequences can be found in most (if not all) bacteria and are scattered around the bacterial genome. These elements can serve as primer sites for genomic DNA amplification. Several families of repetitive sequences have been studied in detail, including the repetitive palindromic (REP) sequence (61,62), the enterobacterial repetitive intergenic consensus (ERIC) sequence (63,64) or the BOX element (59). These can give rise to a PCR product called an inter-repeat fragment. Several studies using primers that target such repetitive sequences have demonstrated only a moderate resolution of this typing strategy among MRSA strains (65-67). Another sort of repetitive sequence analysis by PCR is that of highly polymorphic short-sequence direct DNA repeats in prokaryotic genomes (68). The bordering sequences of these direct-repeat sequences can form a template for PCR primers. The size variation of the amplicon reflects the number of direct-repeats units and can be established by agarose gel electrophoresis (56- 69).

Arbitrarily primed PCR. Arbitrarily primed PCR (AP-PCR) was first described in the early 1990s (70,71). The discrimination level obtained with AP-PCR, also known as randomly amplified polymorphic DNA analysis (RAPD) is based on short primers (10 bp). These oligo's are used under low stringency of amplification conditions. The genetic organization of the bacterial genome among different lineages is reflected by the variable size and numbers of amplified fragments (Fig. 4). The inter-laboratory reproducibility is moderate (72). PCR fingerprinting provides a generally applicable typing procedure for *ad hoc* epidemiological diagnostics and complies with most of the convenience criteria, such as low costs, simplicity and speed.

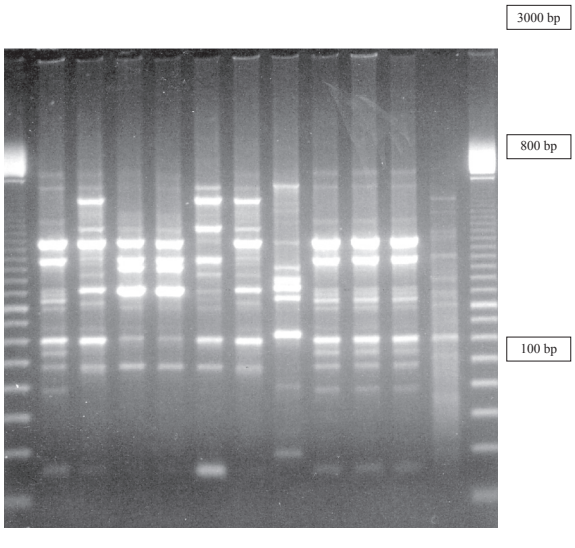

Fig. 4. Characterization of 11 MRSA strains by arbitrarily primed PCR (AP-PCR). The figure represents an agarose gel showing the amplification products from a PCR using a single, random primer. Each lane represents the fingerprint of 1 MRSA strain. The fingerprints are bilaterally flanked by molecular size markers. The sizes of the fragments are indicated on the right.

Amplified fragment length polymorphism analysis. In the mid-1990s, amplified fragment length polymorphism analysis (AFLP) was designed as a typing tool for microorganisms (73,74). AFLP belongs to the category of selective restriction fragment amplification techniques, based on ligation of synthetic adapters, i.e. so-called linkers and indexers, to genomic restriction fragments followed by a PCR-based amplification with adapter-specific primers.

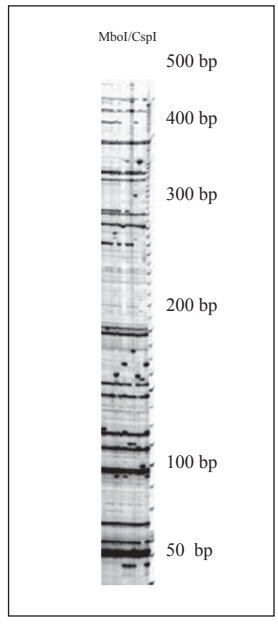

Fig. 5. Representative example of AFLP patterns obtained from MRSA strains. The patterns are the result of template amplification generated after restriction with *Mbo*I and *Csp*I and ligation with sequence-specific oligonucleotide adapters. Selective amplification of some of the fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences, defines the complexity of the fragments. The nucleotides of the primers that cover the fragment are indicated on top of the figure. DNA fragment sizes are indicated on the right.

The amplified products are visualized by DNA electrophoresis (e.g. polyacrylamide gel electrophoresis or capillary electrophoresis, (Fig. 5). To date, the AFLP technique is developed into a highly standardized, robust and automated technique (75) and has the potential for long-term surveillance studies on national and international levels or for analysis of the bacterial population structure.

Pulsed-field gel electrophoresis. Restriction endonucleases that recognize only a few sites in bacterial genomes have been used since the late 1970s. The exposure of DNA to those enzymes yielded large fragments, called macrorestriction fragments, and subsequently were separated by pulsed-field gel electrophoresis (PFGE). During the PFGE procedure, the orientation of the electric field across the gel is changed periodically. The separation of the DNA fragments by PFGE is primarily based on the time needed by the DNA molecules to reorient themselves in this gel, rather than the speed by which they can migrate in it (Fig. 6). PFGE is still accepted as the current "gold standard" for typing many other bacterial species (42, 76-78). PFGE generates complex banding patterns and internationally accepted guidelines for data interpretation were drawn up (79,80). Nevertheless, care has to be taken since the intercenter reproducibility of PFGE remains moderate (81). Recently, diverse multi-national groups cooperated to establish a normalized procedure on the optimization of PFGE and a good level of reproducibility was reached, enabling multi-center comparison of PFGE data (82-84).

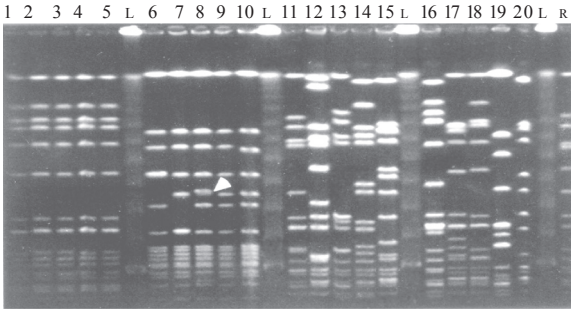

Fig. 6. Representative example of PFGE results obtained after macrorestriction analysis of chromosomal DNA obtained from 5 outbreak MRSA strains (1 to 5) and 5 epidemiologically related MRSA strains (6 to 10). The bacterial DNA is cleaved with the rare-cutter restriction enzyme *Sma*I, followed by a pulsed-field gel electrophoresis. Size markers, indicated as L, were used and the sizes are depicted on the right.

Fourth-phase molecular typing: sequence typing. Comparison of nucleic acid sequences is the most stringent method by which potential relatedness among strains can be defined. However, sequencing of whole genomes is not yet feasible when studying large collections of strains within a species. The challenge for sequence-based typing, therefore, is to identify region(s) within the genome that exhibit variable and conserved sequences that can be sequenced efficiently. An elegant strategy has been the classification of bacterial isolates on the basis of sequences of internal fragments of six or seven so-called housekeeping genes (85). House-keeping genes are conserved genes encoding proteins that are essential for cell viability. For each gene fragment, the different sequences are assigned to distinct allele identification numbers and the combination of the numbers defined for all gene fragments generates the sequence type (ST). Isolates with the same allelic profile can be considered clonally related. Such typing is called multi-locus sequence typing (MLST) (85-87). MLST data can be conveniently stored in a computer and comparison of results between different laboratories is possible via the Internet (86). Housekeeping genes are slowly evolving genes and single-nucleotide polymorphisms within these genes are not discriminatory enough to apply those genes as epidemiological markers in short-term epidemiological issues. Therefore, MLST is thought to be technically very demanding and the technique is more suitable for investigation of the bacterial phylogeny and evolution of population lineages than for typing many strains in hospital outbreaks and epidemics (88).

Criteria for the interpretation of typing results. Theoretically, strain typing simply identifies an outbreak strain and differentiates among non-related strains. In practice, the interpretation of the experimental data leading to correct identification is complex. This is based on technical factors relating to the typing method used or by the fact that an epidemic strain can evolve during an ongoing outbreak and may demonstrate limited genetic variability. A recent study showed that MRSA strains produce PFGE patterns that were relatively stable over periods of weeks to months (89). Interpretation of strain typing results has to distinguish the diverse distances between the strains from the level of micro-evolution (which takes place over days or months during the infectious cycle of a pathogen in a host) within outbreak strains to

major differences among strains as a consequence of macro-evolution (which spans millions of years over global and ecological range of the organism) (90). Interpretation criteria should provide clear guidelines for unambiguous determination of genetic variation level, whether a strain is unique or a component of an outbreak.

The majority of typing methods reviewed here, analyze a relatively small part of the overall bacterial genome. Therefore, identical genotypes have to be classified as "indistinguishable" and not "identical" (91). Tenover et al. (91) translated the number of genetic events into strain (un)relatedness from results, obtained with so- called image- based typing techniques (PFGE, RAPD analysis, RFLP). The same interpretation criteria were applied for MLST typing (92). Essentially, most of the image-based techniques generate complex banding patterns and the interpretation remains speculative. For a more precise definition of strain relatedness, the results obtained with image- based typing systems, can be compared with computer- based software. Analogue peak patterns will be translated into numerical patterns by mathematical calculation. Currently, approximately 200 phylogeny software programs are commercially available, including for instance GelCompar (93), PHYLIP (94), AMBIS (95), Bio-Image (96), Dendron (97), Taxotron (98), Molecular Analyst (99) and Bionumerics (Applied Maths, St-Martens-Latem, Belgium), a biological data analysis software package with a wide variety of applications (100,101). A disadvantage of these bioanalysis software products is the fact that election of bands from fingerprints and normalization between gels has to be done manually, potentially leading to subjective bias by the user. Currently, there are no methods for solving these problems. Full standardization and automation of the performance of a typing system, including the interpretation of the data, could be the solution to circumvent the above mentioned issues. An example of such an approach is the DiversiLab System (bioMérieux, Boxtel, The Netherlands). This system is based on the species-specific amplification of interspersed repetitive DNA elements, present on the bacterial or eukaryotic genome. The fragments are separated with microfluidic chips (Caliper Technologies) and patterns were analyzed with a Bioanalyzer (model B2100, Agilent Technologies, Calif). Comparison of the electropherograms were performed with the DiversiLab software.

Concluding remarks. In the future, microbiological typing and identification procedures that are based on the generation of DNA banding patterns, i.e. the image-based methods, will be replaced by techniques that produce a binary output. These prospective approaches will depend on probe-mediated identification or primary DNA sequence elucidation. Currently, comparative typing methods are used for ad-hoc outbreak studies of limited numbers of strains. Long-term studies, such as continuous surveillance of pathogenic bacteria in specific human populations, require standardized high-throughput methods, the so-called library typing systems, which use a uniform nomenclature (90).

Some image-based typing methods, such as PFGE, have been used for large multi-center studies. Globally, several networks were developed for the validation and characterization of these technologies to obtain inter-center data exchange. The main outcome of these studies was that the optimal procedure has yet to be developed, albeit that MLST turned out to be a very promising candidate technology (88, 92, 102). Research in the near future will have to demonstrate the value of this technology which is currently still very laborious and very technically demanding to most routine diagnostic medical microbiology laboratories. It remains to be determined whether MLST is also suited for ad-hoc nosocomial epidemiological studies. Until then, personal preferences of the researchers involved will remain the prime determinant for the choice of a bacterial typing system.

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