Guidelines for the validation and application of typing methods for use in bacterial epidemiology

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ABSTRACT

For bacterial typing to be useful, the development, validation and appropriate application of typing methods must follow unified criteria. Over a decade ago, ESGEM, the ESCMID (Europen Society for Clinical Microbiology and Infectious Diseases) Study Group on Epidemiological Markers, produced guidelines for optimal use and quality assessment of the then most frequently used typing procedures. We present here an update of these guidelines, taking into account the spectacular increase in the number and quality of typing methods made available over the past decade. Newer and older, phenotypic and genotypic methods for typing of all clinically relevant bacterial species are described according to their principles, advantages and disadvantages. Criteria for their evaluation and application and the interpretation of their results are proposed. Finally, the issues of reporting, standardisation, quality assessment and international networks are discussed. It must be emphasised that typing results can never stand alone and need to be interpreted in the context of all available epidemiological, clinical and demographical data relating to the infectious disease under investigation. A strategic effort on the part of all workers in the field is thus mandatory to combat emerging infectious diseases, as is financial support from national and international granting bodies and health authorities.

CENTRAL THEME
Bacterial typing methods generate isolate-specific molecular fingerprints for assessment of epidemiological relatedness

INTRODUCTION

The ability to quickly and reliably differentiate among related bacterial isolates is essential for epidemiological surveillance, and is an endeavour as old as the discipline of bacteriology itself. Long-standing 'conventional' typing methods, such as bacteriophage typing of *Staphylococcus aureus* and *Listeria monocytogenes* [1,2], serotyping of *Salmonella* spp. and *Escherichia coli* [3,4], or biochemical typing of Enterobacteriaceae [5], have historically been important contributors to our understanding of the natural history and epidemiology of infections caused by strains of these clinically relevant bacterial species.

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Similarly, antibiogram typing has for many years been and, as a matter of fact, still is, in the field of clinical microbiology, a first-line method to identify possible cases of bacterial cross-transmission in healthcare institutions. These methods for bacterial phenotyping have a clear purpose in the confirmation and elucidation of local and national healthcare-associated outbreaks due to bacterial strains [1]. However, although still useful for specific purposes, they have a number of practical limitations which render them unsuitable for comprehensive studies of bacterial population structure and dynamics, and also for the scientifically less ambitious, but very critical, endeavours of infection control and surveillance [6,7]. Furthermore, most phenotypic methods have been developed for specific bacterial species and are not generally applicable. However, although it is generally accepted that phenotyping cannot usually stand alone, in some cases (e.g., serotyping of salmonellae), it is a very useful prerequisite. Nevertheless, the development, application and quality control of phage typing and serotyping are labour-intensive and require skills and methodologies that are difficult to maintain at levels of quality sufficient to satisfy the standards of today's accreditation bodies for microbiology laboratories. More importantly, any given phenotype does not always accurately reflect the genotype of a microorganism, and therefore may not provide a reliable and stable epidemiological marker. The rate of genetic exchange within many bacterial species means that a given phenotype may not always reflect evolutionary history. For example, two isolates that are identical according to phage typing might in fact be quite unrelated, and conversely, two isolates that show quite different phenotypes for a single marker might in fact be closely related. For these reasons, phenotyping has been largely replaced by genotypic or 'molecular' typing over the past two decades [8–13]. In principle, at least, asexual (clonal) reproduction by binary fission implies that genotypic markers should reflect evolutionary history and would therefore be useful in delineating a natural taxonomy. In practice, the ease with which genes can be transferred among different lineages means that the data from multiple markers are required, and even then there is no guarantee that a natural taxonomy will present itself [14]. Polyphasic taxonomy currently uses combinations of different phenotypic or genotypic datasets to define genera, species and even taxonomically relevant subspecies [15–18]. At the same time, however, there are inherently polymorphic loci present in the genomes of all bacterial species that enable further subspecies differentiation. Thus, DNA typing, which essentially comprises the direct or indirect assessment of subspecies nucleotide sequence motifs and their variation in both primary structure and number of copies per chromosome (see Fig. 1 for a generalised scheme), can reproducibly reveal conserved as well as variable characteristics, both at different taxonomic levels and at levels below species/subspecies, the lowest taxonomic rank with official standing in nomenclature.

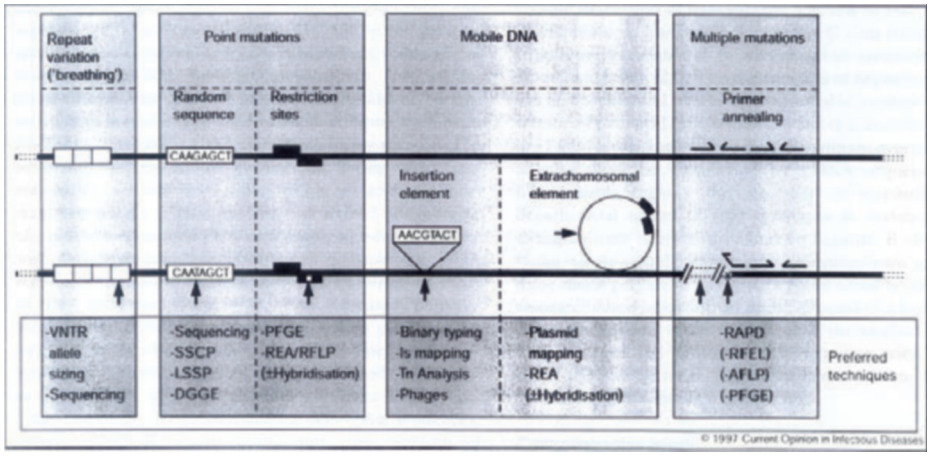


Figure 1. The general features of molecular typing methods. The four boxes show the various molecular concepts associated with genetic variability. Below these boxes, the typing techniques most suited for the detection of such nucleic acid changes are indicated. More technical detail can be retrieved from various sections in the text.

Unfortunately, new molecular typing methods are often proposed for general use without sufficient prior critical evaluation. For example, they may not have been standardised, a minimal number of isolates may have been used for validation, their agreement with epidemiological data may not have been assessed, or the suitability of a specific method–microbe combination for a specific bacterial taxon may not have been addressed [19–28]. Finally, basic terminology—including fundamental terms such as 'isolate', 'strain', 'type' or 'clone'—is often used differently by different workers in the field of bacterial epidemiology.

Here, we present an update of the previous ESGEM guidelines for the correct application of methods and interpretation of the resulting data [29]. We endeavour to define the terminology used in microbial typing, distinguish the major means and purposes of bacterial typing, provide criteria for evaluation, and outline the advantages, limitations and unresolved issues related to the methods currently used. We intend to increase awareness of the importance of methodological evaluations and optimisations, and the appropriate use of control and reference strains, as well as prudent data interpretation. In short, we aim to define the purpose and choice of methods, in combination with interpretation of the results, thereby facilitating the development of practical decision trees. We suggest useful ways for the communication of typing data in general, and more specifically, communication from the laboratory to the clinic. We include discussions on different typing applications and their globalisation, and, importantly, on quality control. Finally, the links between practical baterial typing and phylogeny, population biology and taxonomy are considered. This position paper has been developed through interactions with microbiologists active in the field, and aims to propose genuine and applicable general typing guidelines. These guidelines, however, should always be applied carefully and their consequences interpreted critically in all instances. The intended audience includes, among many others, general and clinical microbiologists, infectious disease specialists, infection control managers, higher degree students, research technologists interested in the molecular epidemiology of bacteria, decision-makers in the context of public health, and workers in reference laboratories.

DEFINITIONS REGARDING ISOLATE RELATIONSHIPS

Bacterial typing has acquired its own vocabulary, in part borrowed from that of other scientific disciplines, including population biology, molecular biology, taxonomy and ecology. Use of this terminology is not always consistent and can be confusing. Prior to presentation of a glossary, we would like to discuss the terms 'isolate', 'strain' and 'clone' in detail, in order to highlight some of the debatable issues concerning definitions, and thereby suggest a more standardised and uniform terminology.

The terms 'isolate' and 'strain' are often used interchangeably, but not always appropriately. A bacterial isolate can be defined simply as a single isolation in pure culture from a clinical specimen. Depending on the state of characterisation, an isolate may be referred to as, for example, 'urine isolate X' (if only the sample type is known) or 'MRSA isolate Y' (if the species and some antimicrobial resistance properties are known). Ultimately, isolates can be characterised as descendants of the same strain. However, there is no agreement concerning the minimal sets of characters required to define any kind of strain. A reference strain is a well-characterised strain that is maintained in pure culture for further study, while a type strain is a special kind of reference strain, i.e., the strain with which the name of the species is permanently associated. An isolate can be assigned to a defined type according to the results of the application of a particular typing method, e.g., pulsed-field gel electrophoresis (PFGE) type X, *spa* type Y. It must be noted that isolates with identical typing results need not necessarily belong to the same strain, since different strains may be indistinguishable with respect to a typing method. The opposite can also be true; isolates with different types may be part of the same (pandemic) strain. This can be observed when the intrinsic evolutionary clockspeed of a given species is higher than average. At present, different nomenclatures for bacterial strains, isolates and types exist and these must be considered with care and used appropriately. To ensure the consistent use of the terms 'isolate'

and 'strain', we suggest the following example: two isolates (1 and 2) can be representatives of one strain (A), but two strains (A and B) can never be the same isolate (1).

The terms 'strain' and 'clone' are also used interchangeably. The 'clone' concept, which is frequently used in the context of bacterial epidemiology and population genetics, also illustrates the importance of correct usage of definitions and nomenclature. 'Clone' is a term coined in the early 20th century in the field of botany and used to denote a group of isolates descended from a common ancestor as part of a usually direct chain of replication [30,31]. The clonal relatedness of isolates is manifested by their display of a significantly higher level of similarity in their genotype and/or phenotype than can be expected for randomly occurring and epidemiologically unrelated isolates of the same species. This epidemiological working definition is less stringent than the definitions of a clone used by microbial geneticists [31–35]. The interest in clones has increased over the past decades, due to the emergence of multiresistant or highly virulent clones of pathogenic bacteria that have become widespread and seem to remain stable for prolonged periods [24–26,33–38]. Ørskov and Ørskov [31] proposed the following formulation: 'The word clone will be used to denote bacterial cultures isolated independently from different sources, in different locations, and perhaps at different times, but still showing so many identical phenotypic and genotypic traits that the most likely explanation of this identity is a common origin.' The opposite of clonality is called panmixis, reflecting free DNA recombination among isolates [35,39,40]. Examples of panmictic bacterial species are *Helicobacter pylori* [41] and *Neisseria meningitidis* [42]. Isolates of panmictic bacterial species tend to display extensive genetic variability, and the molecular fingerprints of a single strain may vary within a limited number of generations.

Since the terms 'isolate', 'strain', 'type' and 'clone' have not always been used according to the definitions given above, we propose definitions of a range of terms that are often used by bacterial typists. We hope that these definitions will contribute to consistent usage among typists and scientists from affiliated fields such as taxonomy and population genetics and dynamics.

GLOSSARY OF TERMS

Some of the general terms defined below have been previously described in the literature [29,43,44]. The internet was scanned via the Google search engine, using the terms as key words (search period November 2006). These definitions may have been adapted slightly to make them consistent with technological and philosophical approaches.

Alert organisms: Bacterial species, strains, types or clones of special epidemiological significance because of their predictable transmissibility and potential for causing difficult-to-treat infections. Identification of such an organism should alert healthcare providers and trigger additional control measures such as barrier isolation of colonised or infected patients. Alert organisms are usually important nosocomial pathogens or organisms with an unusual antibiotic susceptibility profile.

Bacterial epidemiology: The study of the dissemination of human bacterial pathogens, including their transmission patterns, risk-factors for and control of infectious disease in human populations.

Clonal complex: A group of bacterial isolates showing a high degree of similarity, ideally based on near-identity of multilocus enzyme profiles and multilocus sequence types. Clonal complexes are identical to clonal groups.

Clonal reproduction: Mode of, usually, asexual reproduction in which the offspring are essentially identical to the parent. In bacteria, clonal reproduction proceeds by binary fission.

Clone: Bacterial isolates that, although they may have been cultured independently from different sources in different locations and perhaps at different times, still have so many identical phenotypic and genotypic traits that the most likely explanation for this identity is a common origin within a relevant time span.

Cluster analysis: Comparative analysis of typing data collected for a variety of bacterial isolates in order to group the organisms according to their similarity in these data. Clusters can be identified by manual (visual) or computerised methods. The partitioning of a dataset into subsets (clusters) reveals groups that share common traits.

Comparative typing: A typing strategy aimed at assessing relatedness within a set of isolates without reference to other isolates.

Convergence: Independent evolution along parallel paths in unrelated lineages that renders the lineages similar for some trait.

Definitive (library) typing: Type allocation of organisms according to an existing typing scheme aimed at the development of (exchangeable) databases for long-term retrospective and prospective multicentre studies as well as epidemiological surveillance studies.

Dendrogram: Binary tree illustrating a cluster analysis performed on a number of isolates for any chosen number of typing data. Each tree, depending on the cluster algorithm used, depicts possible relationships between the isolates included in the analysis. The basis for the tree is all the pairwise comparisons among the included isolates.

Endemicity: Constant presence in a community at a significant frequency, typically restricted to, or peculiar to, a locality or region. This usually presents as persistent occurrence of disease in a population with a stable long-term pattern of incidence around short-term stochastic fluctuations.

Endemic: Strain present in a given setting over a longer period than if it were epidemic, although possibly at a relatively low frequency.

Epidemic: The occurrence of an organism above the usual endemic level as evidenced by a larger than expected number of infections. Used as an adjective, the rapid and extensive spread by infection and/or colonisation that are widely prevalent, i.e., affecting many individuals in an area or a population at the same time.

Epidemic strain: A strain that is suddenly present in a given setting with an unexpectedly high incidence. (However, it is sometimes difficult to determine whether increased incidence is due to strain traits, since there may well be other explanations, e.g., poor hygienic conditions.)

Evolutionary or phylogenetic tree: A diagram that depicts the hypothetical phylogeny (evolutionary history) of the taxa under consideration. The points at which lineages split represent ancestor taxa to the descendant taxa appearing at the terminal points of the tree.

Fingerprint: A specific pattern (e.g., DNA banding pattern) or set of marker scores (e.g., absorbance values) displayed by an isolate on application of one or more typing methods. These fingerprints may be used for assessment of epidemiological relatedness among bacterial isolates.

Fitness: The performance of a bacterial isolate/strain in a particular environment in terms of survival and reproductive rates.

Genetic drift: The process of random sampling of alleles for each generation, which is relatively important in small populations, and is an alternative evolutionary force for natural selection, causing allele frequencies to change. Genetic drift determines the distribution of alleles in different generations.

Genome: The complete genetic information of an organism as encoded in its DNA and/or RNA.

Genotype: Genetic constitution of an organism as assessed by a molecular method.

Hierarchical clustering: A method that emphasises how adjacent spatial units with high or low disease rates might cluster by ranking the units by disease rate, and then examining how probable cluster adjacencies would be compared to random conditions, and marking off successive clusters wherever low-probability values occur.

Isolate: A population of bacterial cells in pure culture derived from a single colony. In clinical microbiology, isolates are usually derived from the primary culture of a clinical specimen obtained from an individual patient.

Lineage: Group of isolates sharing essential characteristics due to common descent.

Linkage disequilibrium: Non-random reassortment of alleles occurring at different loci due to physical linkage, usually due to lack or inhibition of recombination; strong in clonal organisms and absent in freely recombining populations.

Mutation: The simplest mutation (change) in a DNA or RNA sequence is a point mutation (a one-nucleotide change); other mutations include deletion or insertion of one or more nucleotides.

Niche: A unique environment or set of ecological conditions in which a specific (micro)organism occurs and thrives.

Outbreak: Local, initially small-scale, cluster of disease generally caused by increased frequency of infection in a distinct population (may be caused by single epidemic strains or combinations of different strains).

Panmixis: Situation in which gene exchange occurs randomly in the population at a high rate. Isolates of panmictic bacterial species (e.g., *H. pylori* and *N. gonorrhoeae*) tend to display extensive genetic variability, and absolute fingerprint identity may vary even within limited numbers of generations.

Pathogenicity: Biological ability to cause disease.

Pattern analysis: The process of comparing data patterns generated by one or more typing methods.

Phenotype: The observable characteristics of a bacterial isolate/strain. Primary phenotype markers are the distribution of proteins and other cell components and the morphology and behaviour of cells.

Phylogeny: Evolutionary relationships among members of the same taxon (species, strains, etc.).

Population: A group of organisms of the same species inhabiting a given environment.

Population dynamics: The study of factors affecting the variability of populations of microorganisms over time and space, including the interactions of these factors.

Population genetics: The study of variation in genes among a group of individual bacterial strains, including the genetic evolution of populations.

Selection: A natural process resulting in the evolution of an organism that is best adapted to a (selective) environment.

Species: The basic taxonomic category of bacteria; a named group below the genus level whose members show a high degree of overall similarity as compared with other, more distantly related, strains. There is currently no universally accepted species definition in the context of bacteriology, despite many attempts.

Sporadic: Rare, occurring at unpatterned irregular moments and localities, disconnected in space and time; the opposite of epidemic and endemic.

Strain: The descendants of a single isolation in pure culture, usually derived from a single initial colony on a solid growth medium. A strain may be considered an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic and genotypic characteristics. Cultures of a particular microorganism, isolated at the same time from multiple body sites of a patient and indistinguishable by typing, also represent a single strain.

Taxonomy: Theoretical study of organism classification, which involves the sequential, interrelated activities of allocation of organisms to taxa, their nomenclature and identification.

Type: A bacterial isolate may be allocated to a named type according to an existing typing scheme. Type designations aim at facilitating the handling and communication of typing results, and the development of (exchangeable) databases for long-term retrospective and prospective multicentre studies, as well as epidemiological surveillance studies.

Type strain: A strain, maintained in pure culture, with which the name of the species is permanently associated. The type strain of a species is marked by a superscript T at the end of its identification number. The type strain is simply one of the first specimens of a described species. Unfortunately, many so-called type strains are in fact atypical species representatives.

Typing: Phenotypic and/or genetic analysis of bacterial isolates, below the species/subspecies level, performed in order to generate strain/clone-specific fingerprints or datasets that can be used, for example, to detect or rule out cross-infections, elucidate bacterial transmission patterns and find reservoirs or sources of infection in humans. 'Subtyping', a term commonly seen in American literature, is often used as a synonym for typing.

Virulence: The property of an infectious agent that determines the extent to which an overt disease is produced in an infected population.

WHAT IS TYPING AND WHAT ARE TYPING METHODS?

Pathogenic bacteria replicate and persevere in ecological niches called reservoirs. Reservoirs may be humans, including (fellow) patients and healthcare personnel, animals, plants, water, food and various niches in the environment. Transmission of bacteria from any of these sources may generate clusters of colonisation or infection among humans. Such clusters are recognised mostly as outbreaks of infectious diseases. When these outbreaks are not controlled, major epidemics (due to unrestricted further transmission) may arise. Bacterial epidemiological typing generates isolate-specific genotypic or phenotypic characters that can be used to elucidate the sources and routes of spread of bacteria [46,47]. The scope of typing studies may vary from purely 'clinical' (dissemination of infections from patients, animals or other sources to non-colonised and uninfected individuals) to 'environmental' (the presence or spread of organisms in inanimate surroundings) or even 'industrial' (identification of organisms that are either valuable or a menace to bio-industry). Typing may also be used to identify emerging pathogenic strains or clones within a species, including potential agents of bioterrorism, in forensic biology and as evidence in medico-legal cases. A variety of methods have been developed to generate isolate-specific fingerprints, for epidemiological typing. These methods should facilitate the determination of the relatedness among isolates derived from outbreak situations or obvious and recent chains of transmission, in order to support or reject the hypothesis that the isolates come from a single source.

Typing data should always be considered within the time-frame and current epidemiological context that are being evaluated and from which bacterial isolates have been obtained. For example, more variability can be expected between related isolates when longer time periods are studied. The main focus of data interpretation in the clinical setting would be to identify sources, as opposed to reservoirs of infection or colonisation [48–50]. Thus, typing data can distinguish between cases linked to an outbreak of infections and those unrelated cases due to more complex scenarios. In addition, markers of biological diversity can also be relevant to taxonomy, ecology and the study of pathogenesis.

To put it simply, typing applies distinct labels to bacterial isolates. These labels facilitate identification of transmission routes and sources. However, they can also contribute to in-depth investigations of infectious disease pathogenesis, bacterial population structures and baterial genetics.

Typing can be considered as either comparative or definitive (library) typing. In comparative typing, outbreak-related and unrelated isolates are compared, since comparison of outbreak-related isolates with isolates from the past or the future is not relevant. This is sometimes considered sufficient for outbreak investigation [20]. However, in many outbreak settings, be they nosocomial or community-based, it is often useful to compare strains from a current outbreak with previous strains, in which case a definitive (library) typing method should be used. Therefore, it is important to set up and maintain collections of alert organisms in any typing laboratory. Library systems are those that can be used in different laboratories, by different investigators at various time intervals, with the aim of generating high-quality data to be aggregated in a single database for comparative assessment, in great detail at any time [51]. It is thus important that the typing methods are robust and sufficiently standardised to monitor the organisms of interest. While various multicentre studies aimed at standardising potential library typing methods have been undertaken with varying success, there already exist a number of international networks incorporating databases compiled on the basis of molecular typing data.

Typing can be undertaken at different levels, depending on the situation: locally, at a hospital or other primary laboratory, for small investigations; regionally or nationally, in a reference laboratory, to bear upon wider issues of public health and surveillance; or internationally through collaborative networks, to define or survey the worldwide dissemination of major bacterial clones. At each of these levels, different methods may be applied.

SETTING UP STRAIN COLLECTIONS FOR TYPING LABORATORIES

The initiation and maintenance of strain collections are prerequisites for an epidemiological typing

study. The collection should comprise strains of the species of interest: epidemiologically unrelated strains, sets of strains from outbreaks, and prospective clinical isolates with well-defined inclusion criteria. The number of strains and the complexity of the collection are dependent upon the objective(s) of the research. The organisms should be stored preferably in glycerol broth at −80°C or freeze-dried according to accepted guidelines for strain preservation. Such collections are of much less value in the absence of a(n) (electronic) database of relevant clinical, epidemiological and demographical data concerning the strains attached. Combining typing data with clinical and demographical data is deemed to be extremely important in deriving useful conclusions from infectious diseases surveillance data. The combined data should comprise: strain designation, eventual other designations, species name, the original specimen and its origin, date of isolation, hospital, department, patient code, city, country, and—for external strains—identity of provider. Other relevant (optional) data are: antibiogram, species identification method, and possible association with an outbreak or otherwise. For strategic purposes, it is worthwhile to set up integrated databases linking the hospital information system, strain collection database and typing result database, using appropriate software, either commercially acquired or developed in-house.

REASONS FOR TYPING

Typing methods are used to study the spread and population dynamics of bacteria and other microorganisms in clinical and environmental settings, at levels ranging from a single host to a global ecosystem. To date, these methods are most easily and conveniently applied to haploid organisms [40], but interest in the use of methods for typing of diploid organisms, including parasites, yeasts, fungi and plants, is growing rapidly [52,53]. Finally, space (flight) microbiology and the prevention of bioterrorism are new fields in which microbial typing is useful. In forensic biology, nucleic acid technology is applied to human materials [54,55]. Interestingly, human forensics and microbial typing meet where bacteria can be used to collect criminal evidence or to scan crime scenes [56]. Finally, genotypic methods can also be used in microbial taxonomy.

Surveillance of infectious diseases

Typing methods contribute useful information to epidemiological surveillance of infectious diseases, defined as a systematic, ongoing process of data collection, analysis, interpretation, dissemination of results, and action taken, aimed at recording disease trends and designing ways in which to curb them [48,57–59]. Detection of clusters of defined pathogens (alert organisms) with a similar type may constitute an 'early warning' of a potential outbreak. Library typing, such as serotyping, phage typing, PFGE or multilocus sequence typing (MLST), is mandatory for adequate surveillance of infectious diseases (for examples, see Pitt [20]).

Outbreak investigation

An outbreak can be defined as a temporal increase in the incidence of infection (or colonisation) by a certain bacterial species, caused by enhanced transmission of a specific strain. It has to be noted that outbreaks can also be caused by multiple strains. The increased occurrence of a single strain, therefore, needs to be distinguished from the fortuitous accumulation of sporadic cases. Nevertheless, while this holds true for healthcare-associated infections, it should be kept in mind that in the case of foodborne infections, for example, multi-strain outbreaks can also occur. This situation is one of the many instances when accurate epidemiological and clinical descriptions are needed to prepare the design and corroborate the results of typing.

In this context, typing methods are applied to generate and test hypotheses. Typing determines the number of strains causing the increased incidence and, ultimately, should help identify the source(s) of contamination and the route of transmission. Correct application of bacterial typing will increase the efficacy of control measures aimed at containing or interrupting the outbreak [60,61]. Unfortunately, the relevance of typing in infection control strategies is still under-appreciated. Didactic instructions should, therefore, be provided to those using typing in relation to infection control [62–64]. This should lead to an improved understanding of methodology and a better overall appreciation of the added value of epidemiological typing in the clinical setting. Cost savings can be derived from curbing unnecessary

investigations or control measures when a suspected outbreak is dismissed as an accumulation of sporadic cases derived from a single source.

Study of pathogenesis and the course of infection

We have already briefly mentioned the two major uses of typing in studying infections affecting more than one patient. However, typing can also be used to elucidate the progress of infection in a single patient, e.g., by differentiating between an infection from endogenous microflora and that from an exogenous source [65]. When typing is used to compare groups of strains that are either virulent or non-virulent, pathogenesis-related markers can be identified. Such markers can ultimately be translated into clinically relevant diagnostic targets.

Study of bacterial population genetics

Last but not least, some molecular typing systems may be applied to large numbers of isolates from various origins in order to determine the intra-species population structure, and derive phylogenetic hypotheses from this structure [33–35,66]. For example, PFGE analysis of the *Pseudomonas aeruginosa* genome indicates that the average genomic pattern similarity of unrelated strains ranges between 20% and 60% with an average of 35%, whereas clonally derived strains from a single host cluster at similarity levels above 80% [66,67]. Similarly, high-resolution genomic fingerprinting of *Acinetobacter* has revealed that strains of the same species cluster at 50% similarity or more, while the clone and strain delineation levels are approximately 80% and 90%, respectively [68–70].

The current typing method of choice for performing bacterial population genetics studies, and the one with the soundest biological basis, is MLST [71]. This sequence-based technique has been applied to many important pathogens and has provided valuable information concerning the evolution and diversification of these species. In particular, these data have provided the means to estimate how commonly bacterial genomes undergo horizontal gene transfer and the importance that this process may have for the emergence of clinically relevant strains with heightened virulence or drug resistance [72–75]. Technological aspects of the MLST method will be discussed in more detail in later sections of these guidelines.

CRITERIA FOR THE EVALUATION AND VALIDATION OF TYPING METHODS

Before a typing method may be used in a given situation, its appropriateness must have been clearly demonstrated. Every typing method therefore needs to be evaluated and validated with respect to a number of criteria [76–78]. These can be divided into performance and convenience criteria. Because different investigations may depend on different means and have different requirements, there is no ideal, universally applicable bacterial typing method [8]. Nevertheless, the increasing need to communicate among laboratories and to exchange outbreak investigation or surveillance data requires some degree of agreement on common methods. Such standardisation is, of course, a lengthy and difficult process, but is gradually being undertaken for the most popular and dependable typing methods.

Performance criteria

A good typing method should assess a marker that remains *stable* during the study period, and does not vary to a degree that confuses the epidemiological picture. This marker should be testable in every isolate, i.e., it should provide universal *typeability* of all isolates. It should also usefully *discriminate* among isolates, and this discrimination should be *concordant* with the epidemiological picture. Finally, the results of a good typing method should be *reproducible*, independently of the operator, place and time [79–81]. A high degree of reproducibility will in turn make the results of the method amenable to inclusion in databases and analysis by dedicated computer software.

Stability

This refers to the stability of the markers assessed by the typing method: a strain's marker score should not change rapidly and should correspond with the strain's position in the epidemiological context. For example, the characteristics tested by a typing method should remain stable for each isolate after its primary isolation and during

laboratory storage and subculture. Preferably, the assessment of stability should also be performed in an in-vivo system. Although this may not always be possible, successful examples have been reported in the literature [92]. Because mutations and recombination occur at frequencies dependent upon species, strain and environmental conditions, the stability of the marker(s) tested by each method should be evaluated for each bacterial species studied [93,94]. Stability and reproducibility (see below) are concepts that are sometimes confused. To test stability, multiple subcultures of the same isolate, stored over different periods and under different conditions, have to be processed in the same run to minimise laboratory-introduced variations [95]. A marker can also be considered to be stable if multiple isolates of an epidemic strain obtained from different patients at different moments are indistinguishable by typing based on that particular marker.

Typeability

This refers to a method's ability to assign a type to all isolates tested by it. It can be expressed as the percentage of typeable isolates over the total number of typed (typeable and non-typeable) isolates [82–84]. Whereas most of the genotyping methods can characterise all of the isolates within a population (100% typeability), typeability can be low with classic phenotypic methods such as serotyping, due to the fact that the existing serotyping schemes do not cover genetic variation in full.

Discriminatory power

This refers to a method's ability to assign a different type to two unrelated strains sampled randomly from the population of a given species. It can be expressed as a probability using Simpson's index of diversity [85,86]. Hunter and Gaston's modification of Simpson's index of diversity and fixed confidence intervals are important parameters used for making a decision on strain identity or diversity [86]. The formula used to define the diversity index or, better, Simpson's index of diversity D is:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{S} n_j(n_j - 1),$$

where N is the total number of strains in the sample population, S is the total number of types described, and n_j is the number of strains belonging to the jth type. The index should ideally be 1.00 but, in practice, it should be at least in the order of 0.95 for a typing system to be considered more or less 'ideal'. A 5% probability of error is accepted by most professionals in the field. Calculations of the diversity index should be accompanied by critical assessment of the confidence interval, although this is very rarely done [87]. Typing methods exploring polymorphisms at multiple sites of the whole genome are more likely to be more discriminatory than are methods exploring variation at a single locus. For the purpose of calculation, non-typeable strains can be either excluded or grouped together, although the latter does not imply that they are of the same type. In order to avoid overestimating the discriminatory power of a system, it is best that all untypeables be assembled into a single group.

Epidemiological concordance

The results of a typing method should reflect, agree with, and possibly further illuminate the available epidemiological information about the cases of colonisation or infection under study. For example, epidemiologically related isolates derived from presumably single-strain or single-clone outbreaks should be assigned to identical or related types [22,23]. When validating a method, it is desirable that several sets, e.g., five or more, of outbreak-related strains (n = five to ten isolates per set) are included in the test population (see below). Phenotypic methods are usually less likely to be concordant with epidemiology when, for example, distinct strains display similar phenotypes (due to evolutionary convergence) [96].

Reproducibility

This refers to the ability of a typing method to assign the same type to an isolate tested on independent occasions, separated in time and/or place [88]. The reproducibility of a marker pattern (or data generation in general) and that of type assignment (data interpretation) may be different, and both need to be evaluated. Reproducibility may be influenced by many steps in a procedure, as a result of either the protocol used or the stringency of its application. Factors to consider include: the preparation of materials (e.g., variation in growth conditions, and methods of DNA extraction), different batches or reagents, or

reagent variation as a result of local preparation, different types of equipment, bias in observing and recording the results, and, finally, analysis and interpretation of results. Reproducibility has both intra-laboratory and inter-laboratory dimensions. Both require standardised protocols and adequate personnel training to ensure a reliable method that produces results that are 'fit for purpose' for different organisms in different settings [89–91].

Test population

An appropriate and well-defined test population is a prerequisite for evaluating the typeability, discriminatory power and epidemiological concordance of typing methods. Note that the nature of such a population is, of course, defined by the epidemiological context, the species of organism involved, whether the studies are local, regional or global, and whether long-term surveillance is required. A large test population of isolates correctly identified to the species level (preferably $n > 100$) should be assembled to reflect as much as possible the diversity expected in the species as a whole, or at least in the subpopulation to which the typing method will be applied [20–23]. It is recommended to cover as many ecological niches as may be included in future investigations, such as particular patient populations (including age category, immune status, type of hospital and ward, geographical origin) and relevant environmental reservoirs (e.g., for zoonoses or foodborne and waterborne infections). The test population should include strains that are presumably unrelated epidemiologically, on the basis of detailed clinical and epidemiological data, as well as outbreak-related isolates. For these reasons, it is important that hospital epidemiologists invest in prospective collections of organisms that have given rise to important healthcare-associated outbreaks. The test population is distinct from the panels of control isolates that should be used in many studies. For example, in outbreak investigations, the appropriate level of discrimination of the typing method(s) should be confirmed by comparing the outbreak-related strains to a set of control strains (n = 10–30) from a similar time period, locality and patient population, but which are, *a priori*, not epidemiologically related. We feel compelled to emphasise that, although the earlier version of the current guidelines was published more than 10 years ago, it has not been adopted very widely. Publications in which appropriate test populations are analysed in detail are rare, and the mathematics required to support the corresponding conclusions are hardly ever applied.

Convenience criteria

Once the intrinsic value of a method, as well as its appropriateness for the typing of a specific species, has been established on the basis of the performance criteria discussed above, another set of criteria, those related to feasibility or convenience, need to be considered. These are important for the selection of an appropriate typing method, depending on a number of factors, such as the scale of the investigation, the timeliness required of the results, and the financial and technical resources available. The following criteria of convenience, therefore, need to be considered: flexibility, rapidity, accessibility, ease of use, costs, and suitability for computerised analysis and storage of results [97]. The portability of results is being improved continuously, and this latter criterion is becoming increasingly important.

Flexibility (or spectrum)

This reflects the range of species that are typeable with minimal modifications of the method [98]. The broader the range of bacterial species that can be studied, the more central the position of the method in the general typing laboratory will be. Modern DNA sequence-based methods show optimal flexibility in the sense that the principle, as well as the skills and equipment required, are the same for different species. Nevertheless, these methods still need to be optimised and validated for each species of interest; e.g., amplification primers developed for one species are usually not useful for another.

Rapidity

This refers to the total time required to get from the bacterial isolates to the final typing results. The highest degree of typing rapidity can be attained with methods that are applied directly to clinical materials, the so-called culture-independent procedures [99,100]. Ideally, typing should be performed in 'real time'; having results available within a single working day would strongly

enhance the clinical impact of epidemiological typing in general medicine.

Accessibility
This depends upon the availability of reagents and equipment, as well as the skills required for a given method in a given laboratory.

Ease of use
This encompasses technical simplicity, workload, suitability for processing large numbers of isolates, and ease of scoring and interpreting the results.

Cost
This depends on numerous factors. For example, there is the amount of the initial capital outlay for the equipment, its depreciation, which will depend on whether it is out-of-date compared with newer versions or totally new platforms, the frequency and care with which it is used, and finally, the costs of any modifications to the room. The latter could include the additional options of extra air-conditioning and floor reinforcement. The costs of servicing, the price, need for and ready availability of replacement parts, and the cost of consumable reagents should also be considered. Then there are staffing costs, which will depend on the time required to perform procedures, the number and grade of personnel required, their training and requirements for demonstration of competencies for accreditation or other purposes. These costs can be offset, for example, by income generation, which will depend on the ability to provide typing services for others or income-generating training courses for others to learn the typing method.

Amenability to computerised analysis and incorporation of typing results in electronic databases
These two factors are most important for longitudinal comparison of large numbers of isolates. At the local (hospital) level, data obtained by robust typing methods can be analysed electronically or assessed visually. Visual interpretation, even when only small numbers of isolates are studied, requires normalisation of the data prior to inspection [101]. Nevertheless, since clones are spreading among hospitals or in the community, both regionally and globally, it is important that electronic databases be created, enabling microbiologists and public health institutes to monitor the spread of such strains or clones beyond the hospital level. Of course, computerised analysis is optimal in combination with library methods of typing, with MLST as the current key example.

VALIDATION OF NEW METHOD–MICROBE COMBINATIONS

Application of any typing method requires careful assessment of its suitability for a species not yet analysed by it. New methods or variants of existing ones are published on a regular basis [102], but they vary widely in terms of how well validated they are. It cannot be emphasised enough that testing limited numbers of bacterial isolates without adequate follow-up, using non-validated technology in merely local applications, should be discouraged. In the current era, when complete genome sequences are available for multiple strains of most, if not all, clinically relevant microorganisms, such sequence depositories can generate important clues for the selection of appropriate molecular typing targets. Protocols for frequently used typing methods should be validated according to the recommendations given in this article by networks of expert laboratories. Subsequently, certified 'end-user' laboratories should attentively adhere to these protocols. Admittedly, the latter simple statement is often difficult to translate into practice; the personal preferences of many scientists can severely compromise the objective of working according to a standardised protocol. In conclusion, inter-method validation is important and necessary, both from a theoretical point of view and from a practical perspective [103].

PRINCIPLES AND OVERVIEW OF CURRENT TYPING METHODS

Over the past two decades, a plethora of novel and often innovative typing methods has been developed. These range from methods that assess simple phenotypic traits to DNA sequencing. Previously, the **comparison of phenotypic characters**, which involves the comparison of apparent biological features of isolates, was often abandoned because of the problems with performance criteria already mentioned. Instead, methods involving the **comparison of genomic DNA fragments** were adopted. DNA molecules (or

restriction fragments or amplified sections thereof) can be separated on the basis of their molecular size by gel electrophoresis. Such size comparisons assess differences in the length of DNA fragments obtained from DNA from different bacterial strains. Whether the fragments of DNA are natural (e.g., plasmids) or generated at random, by restriction enzymes or after amplification of the DNA using enzymatic DNA replication (PCR), does not matter; size differences, provided that they are accurately determined, can be excellent markers of strain differences.

By definition, the genome of every bacterial isolate is unique. The mere fact that DNA polymerases make copying mistakes during replication suggests that no genome has a 100% identical counterpart [104]. However, such mutations must be compatible with nature; they must be neutral or at least in line with existing structure–function relationships among the corresponding gene products. Hence, bacterial strains differ with respect to their complete genome sequence, and DNA sequencing methodologies can therefore be used to assess similarity of strains. A challenge for the near future is to assess which DNA sequences are useful epidemiological markers, a task that is greatly assisted by whole genome sequencing [105–107].

Since far more detailed reviews exist concerning the technical aspects of typing methods [50,108], we will restrict ourselves to defining briefly the common aspects and quality characteristics of the methods, without any claim to completeness. The diversity and plethora of methods available to the scientific community are such that it is impossible to be comprehensive in the subsequent sections. Strategic literature references will be included to facilitate and stimulate further reading. Important overviews of typing methods can also be found in several general textbooks on the practical and theoretical aspects of bacterial typing.

Phenotypic typing methods

Phenotyping may involve colony morphology, colour, odour and other macroscopic features, but most typing methods rely on traits that require specialised technology in order to be documented. For example, they may assess, qualitatively and quantitatively, the ability of isolates to grow in the presence of specific substances (be they metabolites, drugs, bacterial toxins or bacteriophages) and their expression of specific molecules (be they surface antigens or allelic variants of housekeeping enzymes). All methods require strict standardisation of experimental conditions, since phenotypes are generally quite susceptible to changes in environmental conditions. In a simple statement: phenotyping results in the grouping of organisms according to their similarity in characters resulting from the expression of their genotypes.

Biotyping assesses biochemical characteristics that are known to vary within a given species. Typeability is usually excellent. Discriminatory power is variable and, to optimise it, a large number of well-selected characteristics, e.g., metabolic reactions, needs to be included in the test scheme. Stability is dependent on the species and characteristic under consideration. The methods are usually technically easy and inexpensive, the data generated are simple to score and interpret, and all tests can be performed, even in the smallest of laboratories, on large numbers of isolates. If reproducibility is demonstrated, it can be used as a library typing method [109,110]. For instance, commercial systems facilitating the measurement of large panels of 'biotype characteristics' have been developed. These systems use versatile redox technologies, enabling the quantification of various biochemical reactions by colour readings [111–114]. The main power of the system lies in its ability to distinguish among strains within a species [115,116]. Phenotype reaction arrays are available and are useful tools in addition to DNA and proteomic technologies. The reproducibility of biotyping is organism- and character-dependent. It is rarely 100%.

Antimicrobial susceptibility testing (antibiogram-based typing) can be performed either by drug diffusion in solid growth media or drug dilution in liquid media using a variety of measurement systems. Most clinical microbiology laboratories perform some sort of antibiogram typing, since its results are commonly used to guide chemotherapy. Therefore, this method has immediate clinical consequences also. Antibiogram-based typing can, with appropriate selection of drugs, be applied to most species. Discrimination is dependent on the diversity, stability and relative prevalence of the detectable acquired resistance mechanisms in study isolates. It is also dependent on the number of

antimicrobials (including antibiotics no longer in use, such as neomycin, which are adequate for revealing specific resistance mechanisms). Testing for resistance to heavy metals (resistotyping), as well as to disinfectants and antiseptics, can provide useful typing information. The utility of this method can vary according to the stability of resistance patterns, which can be insufficient for use as a clonal marker. Some resistance determinants are plasmid-borne and can be readily lost in the absence of selective conditions; in addition, resistance expression can be under the control of complex regulatory systems [23]. Susceptibility profiles expressed as diameters of inhibition zones combined with cluster analysis can provide useful typing data as an adjunct to data generated by other methods [117,118]. There exist large, international databases built around antibiograms, including data on the geographical origin and clinical nature of the isolates. Although these are primarily used to estimate incidences of resistance, they may, of course, also be consulted for epidemiological queries concerning the spread of specific resistance markers [119,120]. It is of note that similar resistance patterns may be due to convergent evolution (as is the case with many extended-spectrum β-lactamase-producing microorganisms, for instance), which is a strongly confounding phenomenon.

Serotyping is traditionally the most important phenotypic method that has been developed from the early days of microbiology. It has led to comprehensive systems for typing of, for example, *Salmonella* and *E. coli* isolates. Most typing sera react with surface antigens. These systems are still widely used in healthcare-associated or food-associated microbiology laboratories. High-throughput procedures using defined sets of polyclonal or monoclonal antibodies have been made available [121]. Typeability and discrimination, complicated by cross-reactions, are variable [8,21,22]. With adequate quality control of both reagent and method, serotyping can be a reproducible, library typing method of wide applicability. Standardisation of preparation and testing conditions is important. Discrimination can sometimes be improved by combining serotyping with SDS-PAGE, resulting in 'western' (immuno)blotting [8,23,122]. Some serotyping schemes (e.g., the one for *E. coli* [4] or M-protein typing of *Streptococcus pyogenes* [123]) are now being replaced by their genotypic equivalents, where variability is assessed at the level of genes encoding for the antigens [124,125]. Similarly, restriction analysis of the amplified O-antigen gene cluster ('molecular serotyping') has proven to be an interesting alternative for classic serotyping of *E. coli* and *Shigella* isolates [126,127]. Genetic instability *per se*, horizontal gene transfer and convergence due to natural or vaccine-driven herd immunity intrinsically limit the power of serotyping methods.

Phage and bacteriocin typing assess the lytic patterns of test isolates that have been exposed to a defined set of bacteriophages, or bactericidal toxins (bacteriocins). These traditional typing methods are restricted to a limited number of species for which such agents have been identified in numbers large enough to provide a useful degree of discrimination. In addition, when new bacterial clones are discovered, additional phages may need to be included in the typing scheme. Types can change over the longer term, and this in itself can be a useful characteristic in endemic situations. Discrimination is therefore variable, typeability often partial, and reproducibility poor. The production and continuous quality control of phages is important, requiring extensive expertise and time-consuming efforts. However, large numbers of isolates can be processed readily, which is not the case with most current DNA fragment-based typing methods. Interpretation of results is not easy and requires training and experience [128,129]. Nowadays, acquisition or loss of phages, which may play a role in virulence, can be traced by molecular typing, providing a modern extension of the role of phage typing [130].

Phage typing has long been an important tool with which to study the epidemiology of *S. aureus* for example, but today it has lost its position as a reference typing method.

SDS-PAGE of cellular and extracellular components can give rise to highly discriminatory typing methods, with applications in taxonomy also [16,131–134]. In the 1980s, these methods were applied to a variety of organisms, but since the 1990s they have been largely superseded by DNA-based methods. Interestingly, the need for comparative analysis of the complex banding patterns obtained by protein SDS-PAGE was the trigger for the development of dedicated computer software that is now successfully applied to DNA fragment analysis. By protein SDS-PAGE,

cell envelope fractions obtained by sonication and stepwise centrifugation, or whole cells, are solubilised in buffer with the denaturing agent SDS and separated under denaturing conditions by PAGE. After staining, the gels are digitised and the images subjected to cluster analysis. If growth conditions, sample preparation and electrophoresis are rigorously standardised, the profiles are reproducible and suited for databases for longitudinal analysis. Protein SDS-PAGE is rather laborious and requires experience; the advantage is that reagents and equipment are relatively inexpensive.

The step from protein SDS-PAGE to lipopolysaccharide (LPS) gel electrophoresis is relatively small, since the samples prepared for protein analysis can be treated with proteinase K, after which they can be used for electrophoretic separation of LPS molecules, followed by silver staining to visualise them. 'Ladder-type' LPS gel electrophoresis can be strain-specific and has been used for comparative typing, but the method is not widely used because it is laborious [135–137].

Multilocus enzyme electrophoresis (MLEE) identifies electrophoretic variants of a set of housekeeping enzymes, encoded by different alleles of the same gene, thus giving rise to small but detectable variations in protein size and charge [138]. MLEE has been used as a reference method for defining the phylogenetic structure of clonal lineages in bacterial populations [33,34]. Although it is neither a rapid nor a widely applied system, it has been very important in shaping the bacterial population biology landscape. Its molecular progeny, MLST (see below), is much more practical and, hence, more widely used nowadays.

Mass spectrometry (MS) is a technique originally developed for the identification of (primarily organic) molecules of a low molecular weight in complex mixtures [139]. Nowadays, the technology can also be used to characterise mixtures of complex biological macromolecules, through their specific degradation products. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS facilitates the generation of molecular fingerprints for entire organisms [140–142]. The method uses intense laser light to evaporate the biological material, which is subsequently subjected to a strong electrical field. Small ions move at high speed and reach a detector before the larger ones. The signals generated are recorded and give rise to complex spectra, characteristic for the molecular content of a bacterial cell. When these spectra are compared using appropriate computer software, bacterial types can be distinguished [143,144]. MALDI-TOF MS is also suited for the analysis of less complex mixtures of, for instance, DNA molecules [145–148] (Fig. 2). Other spectroscopic methods, based on alternative biophysical strategies, can be used as well. Infrared (IR) or Raman spectroscopy are two such methods that can be used for isolate comparison [149–151]. Both use focused illumination of bacterial biomass and record the emission spectra generated. The complexity of the spectrum reflects molecular complexity and, although not every peak in the spectrum can be assigned to a submolecular particle, the composite patterns can allow comparisons to be performed and types to be assigned. Other spectroscopic and chromatographic methods have been commercialised successfully and can provide useful platforms for certain formats of bacterial typing. Gas–liquid chromatography (GLC; the widely used MIDI system) and Fourier-transform (FT)-IR spectrometry/FT-IR microscopy are merely two examples [145,152].

Other methods based on physics approaches will certainly be developed over the coming

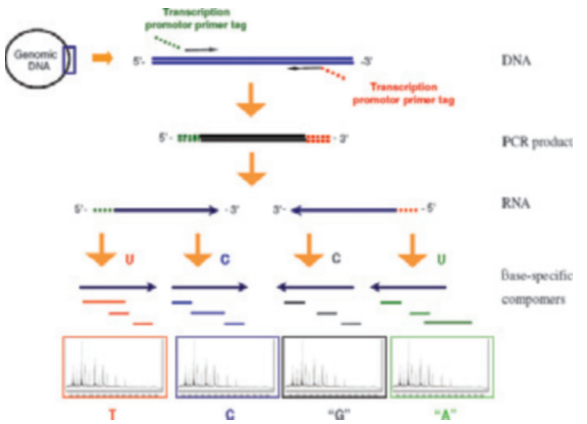


Figure 2. An example of the use of mass spectrometry for the detection of sequence variation in PCR products. After amplification of the DNA stretch under investigation, RNA is transcribed. This is degraded in a sequence-specific manner, and the degradation product is separated and identified by mass spectrometry. This will result in reliable sequence determination. Illustration kindly provided by C. Hönisch (MassCLEAVE; Sequenom, San Diego, USA).

decade. An interesting innovative example is provided by optical mapping of DNA molecules. This method enables one to really visualise DNA fragments of large size and it can already be used for bacterial comparison by looking at single genomic DNA molecules [153]. Another interesting MS approach is the identification of genotypes of bacteria in complex mixtures of clinical samples using MS and base composition [154,155]. It is anticipated that the combination of two-dimensional protein (or DNA) separation techniques, in combination with spectrometric technologies, will open possibilities of new generations of typing systems.

Different ''-omics'' approaches complete the modern phenotyping spectrum. Proteomics collectively describes the methods used for deciphering the protein content of a bacterial cell. These range from 'intelligent' electrophoresis technologies to high-throughput, automated, MS-based protein sequencing facilities.

Glycomics analyses the synthesis, precise molecular features and diversity of polysaccharides, glycans, lipopolysaccharides and other glyco- and lipid complexes, while metabolomics encompasses the diverse metabolic activity of cells. Phenotyping is thus resurfacing with the advent of systems biology approaches [156].

Genotypic typing methods

Genotypic typing methods assess variation in the genomes of bacterial isolates with respect to composition (e.g., presence or absence of plasmids), overall structure (e.g., restriction endonuclease profiles, number and positions of repetitive elements), or precise nucleotide sequence (of one or more genes or intergenic regions). Basic genetic analysis of the molecular event(s) (acquisition, multiplication, mutation, deletion, insertion) associated with pattern variation is the preferred approach to measuring inter-strain relatedness, but is neither always required nor generally feasible [13,157]. A wide variety of genotypic methods has been presented, of which the most widely used will be discussed below in a 'rational-historical' order. The increasing availability of bacterial genome sequences has had, and is still exerting, a great impact on the evolution of these methods, by facilitating the choice of successful typing targets.

Hybridisation-mediated methods.

Direct (and reverse) hybridisation: Direct hybridisation testing of bacterial genomic DNA (without restriction enzyme treatment) is feasible. In all methods, the immobilised DNA to be investigated is probed with DNA molecules that are selective; some templates are recognised, and others are not. The technologies employed vary widely, but the core technology was developed by Southern and colleagues [158] (hence 'Southern hybridisation'). As a recent example, 'binary' typing has been developed for *S. aureus* through the isolation of DNA probes that are specific for some *S. aureus* strains [159,160]. The method proved to be reproducible and easy to perform [161,162]. Similar systems have been developed for other bacterial species [163,164]. Direct hybridisation tests can also be used to define the nature of mobile elements involved in methicillin resistance or to identify determinants of glycopeptide resistance in *S. aureus* [165–167]. The same methodology can be used for typing of DNA amplified by PCR [168] For instance, *Mycobacterium tuberculosis* 'spoligotyping' includes amplification of a locus harbouring tandem repeats with some internal sequence variation. These variants are then identified by hybridisation using repeat-specific DNA probes [169,170].

Ribotyping is a classic variant of a Southern hybridisation-mediated assay [171] that estimates the number of ribosomal gene loci and their position in the chromosome. It is reproducible and applicable to (fast-growing) bacteria, but has a discriminatory power that is usually lower than that of, for example, PFGE [12,26,172]. Fully automated robots for ribotyping have been made available, reducing hands-on time, albeit at a significant price [173,174]. The automated method has been compared with a variety of other genotyping methods [175–182] and, although it was demonstrated to be useful for various bacterial species, it did not always stand out as a superior method [183] since its discriminatory power is relatively limited. Nevertheless, it is robust, and profiles can be compared among laboratories and be used for the generation of databases; hence, it was adopted for some pathogens important in food microbiology [174]. Reproducibility has been documented experimentally during clinical microbiological usage [181,184].

Genome analysis by array hybridisation

Array systems currently represent state-of-the-art hybridisation-mediated testing. This method capitalises on the technological possibility of immobilising up to several hundred thousands of DNA probes per square centimetre of a solid matrix. For most of the clinically relevant microorganisms, whole genome arrays have been developed, based on the available whole genome sequences, and covering all of the genes identified. Probes may be PCR products of defined length, but synthetic oligonucleotides are more frequently used. These platforms facilitate bacterial typing in unprecedented detail. As the method is not yet suited for day-to-day clinical application, careful consideration of target genes is necessary in order to achieve optimal epidemiological concordance. Currently, costs and accessibility also remain problematic. A recent comparison of multiple genomes of strains of the same species has shown that considerable gene variation exists within a species, and the term 'pan genome' was coined to denote the cumulative genome deduced from the individual genome sequences [185]. Hence, it is emphasised that analyses based on single-strain genomes of a given species are not likely to be sufficient to make generalisations about the species as a whole.

Fragment-based methods

Plasmid typing assesses the number size and/or restriction endonuclease digestion profiles, after agarose gel electrophoresis, of these bacterial extrachromosomal genetic elements. It has been used for typing of many bacterial species [9]. Typeability and discrimination are variable, depending on the bacterial species [9]. However, the lack of stability of plasmid content rendered it unsuitable for use as a reliable clonal marker in some studies [186]. It is best combined with other genomic typing methods, to distinguish, for example, between spread of a resistant clone and that of a resistance plasmid [23]. Plasmid typing is still used frequently in combination with testing of antimicrobial susceptibility in modern clinical microbiology laboratories [187,188] to assess whether an antibiotic resistance gene is plasmid-borne and can be transferred.

Among restriction fragment length polymorphism (RFLP) methods, restriction endonuclease analysis (REA) was the first to be widely used. The chromosome is digested by frequently cutting restriction enzymes into several hundreds of small fragments, which are separated by horizontal gel electrophoresis into complex patterns [10]. It is rapid and, under standardised conditions, very reproducible and discriminatory. However, the complex patterns produced complicate interpretation and hinder data exchange among laboratories. In order to simplify the interpretation of REA results, Southern blot and hybridisation steps were added. A variant, which is very important historically, is **ribotyping** (mentioned above), a method that couples genome digestion by a 'frequent-cutting' restriction endonuclease with a 4-bp recognition sequence, and hybridisation with a probe complementary to rDNA. Some of the hybridisation probes used are restricted to a single species; the most illustrious and popular example is **IS***6110***typing** of *M. tuberculosis* [25]. This method has been the agreed standard among tuberculosis reference laboratories worldwide over the past 15 years. It has been applied during hundreds of studies, and its output has been shown to be communicable among institutions and over the years, as thousands of profiles generated in different laboratories have been integrated in a central database [79,189,190].

A new electrophoresis technique, **PFGE**, made it possible to separate large DNA fragments in agarose gels by periodic alternation of the angle of the electric field's direction. These DNA 'macrorestriction' fragments are generated with restriction endonucleases with six or more base pair recognition sites ('rare cutters'), usually yielding fewer than 30 large fragments, normally ranging in size between 20 and 600 kbp. PFGE was originally used for electrophoretic separation of the chromosomes of lower eukaryotes [191], and has enabled epidemiological studies of yeasts and fungi [192,193]. Only in the case of excessive endogenous endonuclease or DNA methylation activities has PFGE been problematic [194]. However, even these technical problems can be overcome by the use of chemical endonuclease inhibitors and alternative restriction endonucleases. PFGE has remarkable discriminatory power and reproducibility, and has therefore become a widely applicable method for comparative typing of almost all bacterial species [8,11,195,196]. With careful standardisation, acceptable levels of interlaboratory reproducibility can be achieved, which have allowed the creation and maintenance of international databases, with the PulseNet effort

representing an important achievement (see also later sections) [28,197–204]. However, 2–4 days are required to obtain results and relatively expensive PFGE equipment is required. Gels need to be analysed closely and carefully, even after digitalisation and computerised processing [205]. To confirm the outcome of the mathematical analysis and to verify, establish or refute finer discrimination, quality control is essential (see also the later section on PFGE data interpretation).

PCR fingerprinting relies on the amplification of genomic fragments flanked by one or two oligonucleotide sequences used as primers. These primers should preferably be cognate to the species being typed (e.g., BOX for *S. pneumoniae* [206] or IS*256* for *S. aureus* [207]). Cognate primers allow for relatively high annealing temperatures, thus contributing to high reproducibility, in contrast to non-cognate primers such as the very widely used 'arbitrary', random-sequence primers that range between six and ten nucleotides in length. Primer pairs are often designed to be directed outwards from repetitive elements, to amplify short spacer sequences lying between these elements. It is a quasi-universal typing method, exhibiting an easily adjustable level of discrimination [13,208]. Its major advantages include flexibility, technical simplicity, wide availability of equipment and reagents, and rapid, same-day turnover. However, interpretation of band differences, of necessity, remains biologically unfounded (it can never be known, for example, if other unobserved 'spacer sequences' existed that were longer than what could be amplified by the DNA polymerase used) and, as suggested, this method can rarely be considered a 'library' method. PCR 'fingerprinting' data, in general, are considered to be non-exchangeable among laboratories [27,195,209,210], although commercial tests claim the contrary [211]. In order to increase the resolution of PCR fingerprinting, an RFLP step is sometimes added. One example of a **PCR-RFLP method** is amplified ribosomal DNA restriction analysis (ARDRA), which has been used successfully for species identification of various organisms, including acinetobacters [43,212], while there are numerous examples of this methodology for typing of other bacterial species. Essentially, PCR-RFLP monitors for a variety of mutations that can occur in restriction sites and, as such, is a variant method for detection of single nucleotide polymorphisms (SNPs) (see later in 'Sequence-based methods'). Yet another PCR-based typing method is **amplified fragment length polymorphism (AFLP) analysis**. **AFLP**^{TM} is the patented name of a method designed to selectively amplify subsets of genomic fragments generated with one or two restriction enzymes, usually a 'rare' and a 'frequent cutter' [213,214]. After ligation of adapters to the restriction fragments, selective amplification is achieved by the use of primers that consist of the adapter-derived core sequence, including the 3'-part of the restriction half-site, and an extension of one or more selective bases. Elongation will only take place if a nucleotide complementary to the selective base in the primer sequence is present in the fragment. Products can be separated in agarose gels [215–217], but usually one primer is labelled and fragment separation is obtained using an automatic DNA sequencing instrument with automated data capture. The digitised and complex DNA fingerprints are generally highly reproducible and have been used very successfully for the high-throughput molecular typing of large numbers of bacterial isolates [218,219] (Fig. 3). Essentially, nearly whole genome coverage can be attained.

For some bacterial species, databases have been developed and inter-centre reproducibility assessed [220–222,224]. Recent and, as yet, unpublished studies have revealed that AFLP may also suffer from the absence of inter-centre reproducibility, especially when different electrophoresis platforms are being employed [220,221,223]. For *Acinetobacter* spp., as illustrated in Fig. 4, the method is useful to identify species [217], clones within *Acinetobacter baumannii* [69,118] and epidemic strains [218]. The profiles generated with labelled primers and automated sequencing equipment are highly complex, and dedicated software for cluster analysis is therefore mandatory (Fig. 4).

Multilocus variable number tandem repeat (VNTR) analysis (MLVA) is also a PCR-based typing method that capitalises on the inherent variability encountered in many regions of repetitive DNA. Repetitive DNA is often incorrectly copied in bacterial species, through slipped strand mispairing (SSM) [225–227], thus resulting in shortening or lengthening of the repeat region due to deletion or insertion of repeat units,

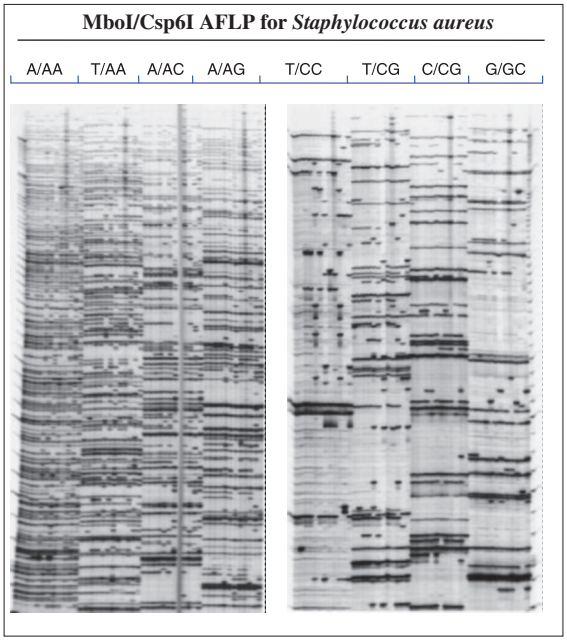


Figure 3. Example of high-throughput amplified fragment length polymorphism analysis for 12 strains of *Staphylococcus aureus*. For the same set of strains, various selective primer pairs were employed, the terminal, selective sequences of which are identified on top of the lanes. Courtesy of G. Simons (Pathofinder, Maastricht, The Netherlands) and H. Witsenboer (Keygene, Wageningen, The Netherlands).

respectively [226]. This type of DNA variation can be simply assessed by performing repeat-spanning PCRs and determining the length of the PCR product. In the case of large repeat units, the analysis system can be simple (e.g., agarose gel electrophoresis). However, for shorter repeats, more complex electrophoresis or MS methods are required. For each repeat locus, a digit can be assigned, representing the number of repeats implied (by electrophoresis) or demonstrated (by sequencing). When assessing the length of the product, normalisation of the migration distances is required to guarantee accurate length measurement (Fig. 5). When several repeat loci are analysed per isolate, several such digits are obtained, resulting in a multi-digit, specific strain code [226]. Dedicated MLVA systems have been developed for a variety of species [227–236]. When compared with other genotyping methods, MLVA has, in general, performed well [229,237,238]. However, few multicentre studies have been undertaken. Given its techical simplicity, MLVA may have a successful future. The major drawback is that the evolution of repetitive DNA may be too rapid, compromising epidemiological concordance. When the mutation frequency in a locus is known and the frequency of certain alleles in a population is documented, it is possible to calculate whether two isolates are identical on the basis of chance. This is not feasible with other fragment-based methods. Also, as for all of the methods that rely on the estimation of molecular size based on standard curves, the accurate sizing of fragments, even using fluorescent detection systems, is not a simple task, as it is mobility dependent on sequence composition as well as length.

Sequence-based methods

Single-locus sequence typing (SLST) is an umbrella term for a variety of methods, in which sequencing of a single genetic locus has been shown to provide valuable typing results. Analysing a single locus means that the amount of DNA to be sequenced is limited, but it is imperative to select gene sequences that are (highly) variable. The best example of an established, epidemiologically significant SLST scheme is that of *emm* typing for *S. pyogenes*, which is the 'genotypic descendant' of M-serotyping, and relies on DNA sequencing of only 150 nucleotides, coding for the N-terminal end of an isolate's M protein [239]. An international database, incorporating a query module, ensures the continuing enrichment of the type repertoire, and already includes over twice as many types as those acquired through the previous use of antisera (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Another more recent example is that of the *S. aureus* protein A gene, *spa*, whose repeats are variable in number and individual sequence [239]. This feature formed the basis for the currently used sequencing system, which has been further elaborated upon and validated [240,241] (Fig. 6). The development of dedicated software and the possibility of determining sequences rapidly have now led to an automated system, which is 100% reproducible among different centres [242–244]. In the case of typing studies performed on the basis of DNA sequences in hypermutable regions, it should be noted that generation of variation may exceed the speed of spread; mutants may arise during an outbreak and thus falsely suggest that the outbreak has multiple sources rather than one.

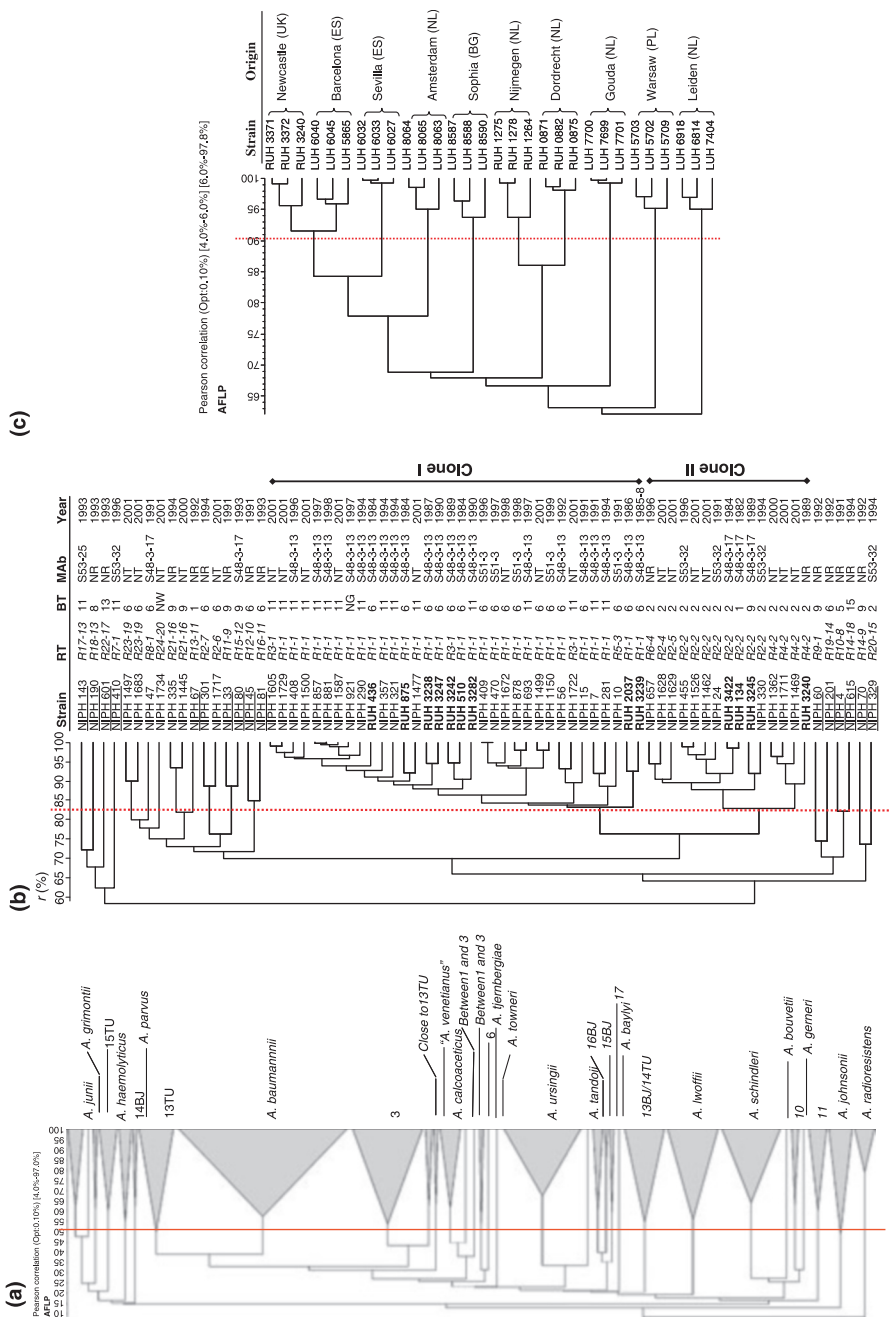


Figure 4. Amplified fragment length polymorphism analysis of *Acinetobacter* strains to delineate species, clones and strains at different similarity levels. (a) All currently described species cluster at >50%. (b) European clones I and II of *A. baumannii* cluster at ~80%. (c) *A. baumannii* strains of the same outbreak cluster at ≥90%.

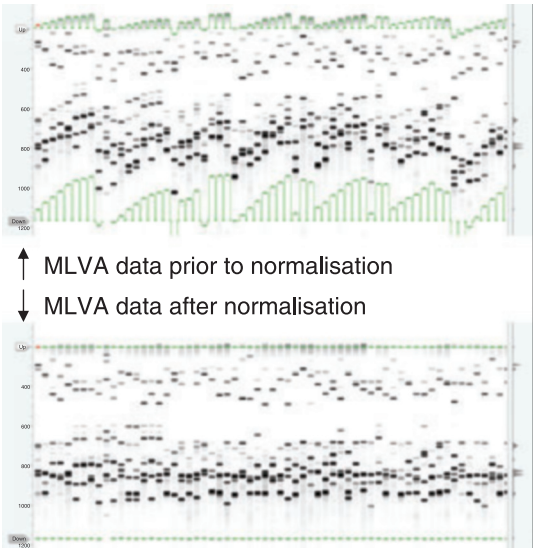


Figure 5. Data normalisation of multilocus variable number tandem repeat analysis (MLVA) data by stratification for two molecular weight markers. Illustration kindly supplied by P. François (Geneva, Switserland). Note that the marker-based normalisation 'flattens' the picture, making the data more comprehensible. As can be seen by the dramatic changes in the profiles, internal migration controls and standardisation are extremely important.

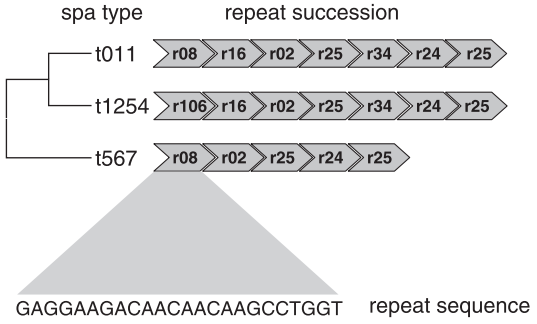


Figure 6. Principles of *spa* typing of *Staphylococcus aureus*. Part of the protein A-encoding gene, containing 24 nucleotide repeats, is amplified and sequenced. Sequencing reveals the primary structure of the repeat units, which facilitates identification by an r-code. On the basis of the series of r-codes identified, a *spa* type (t-code) can be defined. On the basis of t-code relatedness, the homology score among types can be calculated. In the example above, types t011 and t1254 are more closely related than either of the two to t567. Courtesy of A. Mellmann, University of Muenster, Germany.

MLST is the genotypic descendant of MLEE (see above) and assesses DNA sequence variation among the alleles (usually five to ten) of housekeeping genes [71]. It has been widely accepted and constitutes one of the major 'typing successes' of the past decade. It is very important to note that the 'wet lab' developments were paralleled by very important efforts to standardise the interpretative, free software (e.g., eBURST) and to make data freely available via the internet [245–248]. The implications for population genetics and dynamics may be more significant than those for bacterial epidemiology, since polymorphism in the slowly evolving genes, which are its targets, may not be high enough for useful epidemiological comparisons. Furthermore, the genes in question are unlikely to have any direct relevance to virulence or drug resistance traits [249–250]. Other methods using non-housekeeping genes, or using a combination of housekeeping genes and those under presumed selective pressure, have since been described (http://www.mlst.net; http://web.mpiib.berlin-.mpg.de/MLST/; http://www2.pasteur.fr/-recherche/genopole/PT8/MLST/). This is currently the proposed method of the European Working Group for *Legionella* Infections (EWGLI) for epidemiological typing of *Legionella pneumophila* [87,251]. MLST has indeed led to many studies analysing bacterial population genetics, resulting in the successful identification of major sequence types (STs, clones) and clonal complexes (CCs) of clinical relevance in a wide variety of species [75,251–254] (Fig. 7). The results, strings of digits representing different alleles, are easily and unequivocally exchangeable, much more so than images of electrophoresed DNA fragments, for example. This already facilitates the development of publicly accessible databases for comparison of sequence typing results [255,256]. In combination with novel sequencing protocols, this method will probably remain the most popular for bacterial population geneticists in the years to come [257]. Nevertheless, it has practical disadvantages, including limited accessibility and high cost [258]. The practical use of MLST in the field of clinical microbiology has to be accessed, as well as whether it will allow the clinical microbiologist to draw conclusions about the spread of strains in a restricted time-frame.

SNP genotyping involves the determination of the nucleotide base that is present in a given isolate at defined nucleotide positions known to be variable within the population. In essence, MLST is an SNP genotyping method, but it is applicable only in genetically heterogeneous species where many SNPs are located within the gene

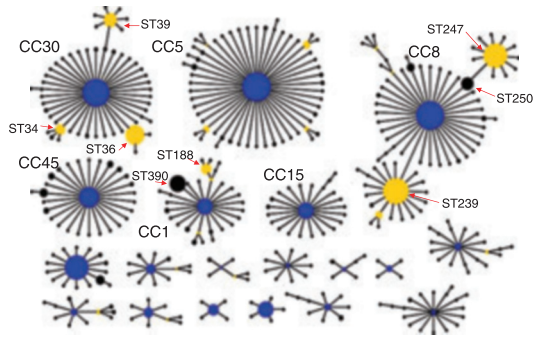


Figure 7. The major clonal complexes of *Staphylococcus aureus* as defined by multilocus sequence typing (MLST)/eBURST. The figure was generated using eBURST on the whole *S. aureus* MLST dataset, consisting of 1688 isolates (832 sequence types (STs)) as of October 2006 (http://saureus.mlst.net/eburst/). Singleton isolates and minor clusters were removed, and the remaining clonal complexes (CCs) arranged for clarity. Each circle represents one ST. The diameter of the circle reflects the frequency of that ST (i.e., the number of isolates). Linked STs differ at one locus out of the seven (single-locus variants (SLVs)). For each complex, a 'founder' ST is assigned, which is the most parsimoniously 'central' ST (shown in blue). 'Subgroup founders', which are STs from which at least two SLVs have descended, are shown in yellow. Six major CCs are named (CC30, CC5, CC8, CC45, CC1, CC15)—in each case, these names refer to the ST of the founder (e.g., the blue founder of CC30 is ST30). Other common STs of clinical relevance are indicated by the red arrows (e.g., ST36 consists of EMRSA-16 strains). The arrangement of the CCs does not reflect the relatedness among them.

portions considered. Strictly speaking, SNP genotyping refers to the analysis of nucleotide polymorphisms that are rare (e.g., less than one in 300 bases) along the bacterial chromosome, rendering the direct assessment of the base identity at the variable position much more efficient than direct sequencing of the surrounding region. Hence, SNP genotyping methods are primarily applied to define the relationships among isolates of homogeneous pathogens such as *M. tuberculosis* [259,260], *Bacillus anthracis* [261], *E. coli* O157:H7 [262] or *Salmonella enterica* serotype Typhi [263].

Variable positions that are useful for typing or phylogenetic analysis must have been discovered prior to the application of an SNP genotyping method. Mutation discovery can be achieved by genome-wide approaches such as shotgun sequencing of several strains [264] or microarray hybridisation-based comparative genome sequencing [262,265]. Polymorphisms can also be revealed by screening a number of defined target genes via sequencing, as in MLST-like approaches or faster approaches such as denaturing High Performance Liquid Chromatography (dHPLC) [263]. In order to obtain a set of SNPs that would represent the diversity of a species in an unbiased manner, it is crucial that mutation discovery be performed on a set of strains that are representative of the breadth of diversity and phylogenetic lineages of that species. Indeed, sets of SNPs derived from comparison of strains that are representative of a limited number of lineages will mostly contain those SNPs that accumulated during the evolution of these lineages (but will ignore SNPs that appeared in other lineages), a phenomenon called 'discovery bias' [259,266].

Once a set of SNPs has been selected, a given bacterial sample can be screened by a variety of SNP genotyping methods. Direct sequencing of regions encompassing the SNPs either by Sanger sequencing or by pyrosequencing is easy to implement and reliable for determining the base present at the targeted SNPs [267]. However, as stated above, this approach is not efficient if the entire sequenced region contains only one or several SNPs. SNP genotyping methods, designed to be applicable to a high number of SNPs and samples, are currently being developed at a fast pace, essentially because of the need for high-throughput SNP genotyping in human diversity studies and pharmacogenetics. For example, the method known as 'mini-sequencing' involves the use of a mixture of all four dideoxynucleotides (without deoxynucleotides) to extend a primer by a single base. The identity of an SNP can be determined by using a primer ending just one base upstream of the SNP. The incorporated base can be determined by fluorescence after capillary electrophoresis [252] or by direct measurement of the mass of the resulting product by MS [268]. Several reviews describe promising SNP genotyping approaches [269–273].

Similar to the fact that using the same target genes in MLST studies allows international standardisation and comparison of genotyping data from different users, the use of standard sets of SNPs for given bacterial species or groups should facilitate future collaboration.

INTERPRETATION OF TYPING RESULTS

Theoretically, the ideal method with which to define the genetic relatedness of bacterial isolates

at the subspecies level would be complete genome sequencing [8]. Nevertheless, even when it originated, the thresholds of epidemiologically useful discrimination would have to be debated according to, not only the species, but also the requirements of each epidemiological study setting. Therefore, several less comprehensive, but more practical, methods are now used to assess polymorphism in bacterial genomes, as outlined above. The data thus generated raise questions about interpretation, which is often complex. There are several general issues, however, that are related to the quantitative analysis of bacterial genomic polymorphisms obtained for typing. A number of ways of assigning types, building on previously published suggestions and the accumulated experience of numerous 'typists', are proposed below.

Interpreting DNA fragment patterns

Isolate relatedness is frequently inferred from genomic typing methods on the basis of DNA fragment size after separation by electrophoretic methods, in terms of either absolute number of band differences or percentage similarity of banding patterns. Percentage similarity scores are generally preferred because they are independent of fingerprint complexity, require simple mathematics and can be generated by dedicated software programs. In addition, on the basis of percentage differences, categories of strain relatedness can be defined in a concise manner. However, percentage similarity will be influenced by the level of tolerance in the differences in band position chosen for each analysis.

The absolute number of band differences is a measure that needs to be interpreted with caution; its weight will be related to the denominator, which is the number of resolved DNA fragments. This number is related to inherent intra-species genome variation rates, the choice and number of genomic sites probed (itself depending on the number and nature of restriction enzymes and/or primers or probes used) and on the amplification and/or separation conditions. Thus, genomic pattern similarity values must be based on a sufficiently large number of genomic sites/bands for each isolate. If low-copy-number and variable-copy-number RFLP probes (e.g., IS sequences) are used, a composite similarity coefficient must be constructed by adding the data obtained using multiple probes. In addition, any inferred measure of inter-strain relatedness is relative only to the overall relatedness in that particular sample of isolates. At any rate, genomic pattern similarity can in no way be considered as a measure of genetic distance, because band positions are not independent, and nor are they evolutionary units. Nevertheless, in practice, a concise set of simple rules for interpretation is obviously useful.

Assigning types by interpreting PFGE-generated patterns

When compared by PFGE, two isolates differing by one mutational event (from a single nucleotide substitution to insertions or deletions of longer DNA sequences) may differ by zero (when the mutation alters neither a restriction endonuclease site, nor the size of the resulting fragments—as a single nucleotide mutation outside the restriction endonuclease recognition site may do, for example) and up to four DNA fragments, or 'bands' [274]. When there are no observed band differences, the isolates should be termed 'indistinguishable', rather than 'identical', and assigned to the same type (e.g., A) and subtype (e.g., A1) if other subtypes exist. Such subtypes (e.g., A1, A2, A3) will be assigned to isolates that differ by one to four bands (Fig. 8). According to a similar calculation, five to eight band differences could be attributable to at least two mutational events. It has been proposed that isolates putatively distant by two mutational events should also be assigned to subtypes, and that only isolates distant by three or more mutations (therefore differing by at least nine bands) should be assigned to distinct types (e.g., A, B, C) [90,273]. Tenover *et al*. [90] furthermore suggested that, in the context of habitual healthcare-associated outbreaks of limited duration (usually restricted to episodes of half a year or less), isolates differing by one to four bands be considered as 'closely related' and therefore 'probably part of the (same) outbreak'. In short-term outbreak typing, single band differences should be deemed important. Isolates differing by five to eight bands would then be 'possibly related' isolates and therefore 'possibly part of the outbreak'. Unfortunately, these suggestions are often misinterpreted as hard and fast 'guidelines' and are applied outside the context that the authors took great pains to delineate. We therefore wish to emphasise that epidemiological

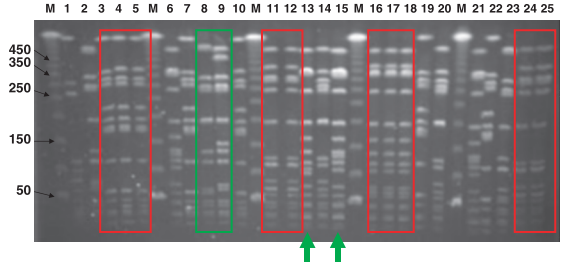


Figure 8. Example of a pulsed-field gel electrophoresis (PFGE) analysis. Lanes marked M display molecular weight markers, the sizes of which are indicated on the left. Note that for normalisation purposes, markers are used every sixth lane. That such precautions are required is made obvious by the electrophoretic anomalies that can be observed in lanes 1–3, where the fragments are not exactly vertically aligned. Red boxes identify indistinguishable patterns, whereas the green box identifies a pair of related patterns differing by two bands, thereby tracing subtypes. This suggests an insertion deletion event in one fragment or the presence of extrachromosomal elements that differ in molecular size. Gel picture provided by D. Horst-Kreft (Erasmus MC, Rotterdam, The Netherlands).

interpretation of differences in PFGE patterns cannot blindly follow these suggestions. Examples of possible exceptions and points to consider follow.

The relative validity of this simple 'biological rule' becomes immediately apparent if one considers that, according to the above, four band differences may arise from four independent mutational events, each giving rise to only a single band difference, while five band differences may arise from only two mutational events. In this case, two isolates with the former relationship would be less 'related' than two with the latter. Once again, only the consideration of epidemiological data would help to clarify the issue. On the other hand, even strains differing by a single band difference may have distinct biological and epidemiological characteristics. For example, during an outbreak of *S. enterica* subsp. *enterica* serotype Blockley that lasted for several months, two PFGE subtypes, named A2 and A4, differed not only in their resistance to nalidixic acid, but also in their temporal and geographical distribution [274]. In this case, the outbreak was extended, and more band differences may have been expected. However, it needs to be borne in mind that the isolates belonged to the same serotype of the same subspecies of the same species. Therefore, the inherent diversity of the study sample was already limited, which makes it clear that it is, indeed, important to take the evolutionary mutation rate of a species (if known) into account.

The following recommendations follow from the above discussion:

1. Isolates with patterns differing by one to four bands should be assigned to subtypes of the same type.
2. Isolates with patterns differing by five or more bands should be assigned to distinct types.
3. Inferring the epidemiological relationship of two or more isolates, according to PFGE types or subtypes, requires careful thought in every case, and consideration of the contribution of other information (clinical, epidemiological and biological characteristics of the outbreak and the possibity of invader isolates being introduced during the outbreak).

The foregoing applies to visual analysis of a usually limited number of profiles. Computer-assisted cluster analysis based on the similarity of profiles requires the prior decision of a cut-off similarity level. The similarity of PFGE profiles requires that only positional correspondence is taken into account; the Dice coefficient is the most widely used for this purpose. Computer-assisted cluster analysis is inevitable for the comparison of large numbers of profiles generated at different moments and—in the case of inter-laboratory networks—at different locations. A certain 'similarity threshold' has to be chosen to define types in this situation [67]. An inter-laboratory study with a rigorously standardised protocol investigating outbreak and non-outbreak strains of *A. baumannii* showed that strains regarded as being the same type clustered at 95–100% if processed in the same laboratory. Central analysis of the data of the same set of strains generated by the three participating laboratories showed that strains of the same type clustered at 87% [28]. Identification of similar or identical strains (types) by band-based pattern analysis at a similarity level of 80% was also instrumental in delineating a clone of multidrug-resistant *A. baumannii* in Southeast England [276]. Since percentages are usually calculated using software programs in which parameters (such as the tolerance of band differences) can be set by the user, the same patterns may yield different quantitative relationships. Furthermore, the user must always check the software assignations, since, for example, gel imperfections may be interpreted as bands by the program. Finally, when two or more

gels are compared, imperfect reproducibility of electrophoretic conditions may lead to, for example, systematic band shifts, which again the software program might interpret as differences, while the careful user can see them for what they are. Confirmation of software groupings according to the user's critical eye, and judgement based on additional information, are therefore essential at all times. In conclusion, the following recommendations apply to assignment of types using algorithm-generated percentage differences (valid for all of the 'band-based' methods): (i) in the event that the 'biological rule' described above fits the algorithm-generated grouping, the aforementioned recommendations apply; (ii) in the contrary case, i.e., when isolates differing by five or more bands cluster together in epidemiologically plausible groups, it is advisable to assign 'clusters of similarity', rather than 'types'.

As a final comment regarding interpretation of PFGE-generated patterns, it is emphasised that large studies assessing inherent intra-species variability would have allowed a more rational design of rules for the assignment of types. Unfortunately, such studies have not been undertaken in large numbers. However, most of the European national health centres have developed various typing databases containing hundreds, if not thousands, of molecular fingerprints. For example, databases comprising over 1200 different fingerprints have been developed for the agent of whooping cough, *Bordetella pertussis* [277,278].

Assigning types by interpreting PCR-generated patterns

Type assignation to PCR-generated band patterns by visual analysis is even more problematic than it is with PFGE-generated patterns, since the biological explanation of band differences is usually unclear, and at any rate complex. In addition, it may be tempting to take band intensity into account. Thus, recommendations in this case would have to be limited to the following:

1. Isolates differing by one or more bands should be assigned to distinct types.
2. Band intensity should only be taken into account once it has been demonstrated unequivocally, by appropriate replicate experiments, that it is reproducible.

However, essentially the same requirements as those for PFGE apply to the inference of strain relationships from PCR-generated types that are based on the comparison of banding patterns (e.g., AFLP). As discussed, when assessing MLVA by gel electrophoresis, some information (e.g., point mutations) may be lost, in contrast to analysis by DNA sequencing. MLVA-generated banding patterns should therefore be interpreted as though they were PCR-generated.

For computer-assisted pattern analysis of PCR- and AFLP-generated fingerprints, the Pearson product moment correlation coefficient is the most objective and reliable similarity measure. It (i) is independent of relative intensities of patterns; (ii) is largely insensitive to differences in background; and (iii) does not suffer from subjective band detection and band-matching criteria, since it compares the entire profile rather than specific band characteristics and relative band intensities. However, for simple PCR-RFLP-generated profiles, a band-based coefficient such as Dice is therefore recommended.

Generally, if groups are robust, different similarity measures and clustering algorithms may largely reveal the same grouping patterns.

Analysis of MLVA profiles

Analysis of MLVA profiles in potential outbreak situations, as with other methods, is best informed by detailed population studies that have been performed previously. These will indicate the likelihood of a change in repeat number at a particular locus during the timeframe of an outbreak. In most situations, isolates within an outbreak will (or should) have an identical MLVA profile. However, whether this profile is common in isolates unrelated to this outbreak, i.e., the background distribution of the MLVA profile in the bacterial population as a whole, is critical information. Also essential, particularly with respect to microsatellites, is the fact that alterations in repeat numbers occur so rapidly that the profile could change during the course of an outbreak. Although MLVA schemes have been proposed for many bacterial pathogens, the prerequisites listed above have seldom been met [278–280].

MLVA can be used for population studies of microorganisms. An approach that is frequently applied to MLVA profiles is an implementation of the minimum spanning tree, based on the same

principles as the eBURST algorithm. These approaches yield maps of predicted relationships among strains on the basis of single-locus (where the profile varies at one locus) and dual-locus (where the profile varies at two loci) variants. If it can be assumed that variation in repeat number at a particular repeat locus is stepwise, i.e., an isolate with six copies of a repeat at a given locus is more closely related to an isolate comprising 'five repeats' than one comprising 'four repeats', distance methods that take the repeat number into account can be applied. However, if this assumption cannot be made, categorical approaches that consider all allelic numbers as equally distant are appropriate.

Interpreting differences among DNA sequences

As mentioned previously, MLVA and sequence-based methods are likely to replace band-based methods, mainly because of the difficulties in comparing banding pattern profiles among laboratories, despite use of common protocols. Sequence-based methods are certainly more portable, as the data are comparable regardless of the platform used to generate them. The only prerequisite is that the data are of adequate accuracy. The value of any sequence database is determined by the quality of the data within it, and the role of the database curator is therefore very important. Most major sequence databases, e.g., MLST.net, require submission of the raw sequence trace files from the laboratory when a new allele type is proposed [281]. A personal check of the data by the curator, before accepting the submission, will ensure that the apparent new allele type is not due to an error in the DNA sequence. Although software (e.g., Phred/Phrap) can assess DNA sequence quality of individual traces or of contigs [282], most curators believe that manual curation remains the reference standard (PulseNet and SalmGene are the best known representatives of this category). The *L. pneumophila* SBT database [87], accessible via the EWGLI website (http://www.ewgli.org/), uses a combination of automated sequence quality checks and manual curation. If DNA sequences are submitted to a database in text format, no guarantee of the quality of the data is given, beyond checking for ambiguous bases (the presence of non-A, G, T, C, such as N, R, W, Y), which may indicate that the original sequence was not optimal. If the target used for typing is a coding sequence, it can be confirmed that the open reading frame involved is not abrogated. As DNA sequencing is increasingly used in clinical applications, rigorous checking of the quality control processes that curators of such databases adopt is recommended.

International efforts in standardisation of type nomenclature and typing protocols

International travel, migration and food commerce are the main factors that have contributed to the worldwide spread of bacterial clones. Therefore, the need for international databases, including those with typing information concerning epidemiologically relevant strains, is strong. Building such databases, in turn, relies upon standardisation of typing methods, and on regular quality assessment ring trials for all participating laboratories, to guarantee consistently comparable data. Currently, two types of such databases have been developed. First, there are international catalogues of prototype strains, e.g., MLST.net and the SeqNet.org *spa* sequence repository (spaserver.ridom.de). Second, there are the molecular epidemiology databases. These include typing data and information concerning the clinical and/or epidemiological features associated with the isolates analysed (e.g., PulseNet and SalmGene).

Inter-laboratory 'ring trials' are a relatively recent development, spurred on by the need for reliable data to be used in international surveillance. Unwillingness on the part of laboratories to abandon methods that have taken time and effort to develop and that produce good results hinders the standardisation of methods. A way forward is to first seek harmonisation rather than standardisation, changing only those aspects of a protocol that are shown to be critical to intra- or inter-laboratory reproducibility. An example of this was the HARMONY project [203], where PFGE protocols were examined in several laboratories. DNA preparation methods, for example, were not found to be an important factor, provided that all methods produced good-quality DNA. Many other aspects required direct standardisation, however. Pulsing conditions were particularly critical and, by comparing gel results among laboratories, new electrophoresis conditions that

produced optimal separation within an acceptable run time with good inter-laboratory reproducibility were agreed. Several laboratories have also found the protocol to be ideal for analysing coagulase-negative staphylococci, thus reducing the number of mandatory laboratory standard operating procedures (SOPs).

Several validated databases exist, making it possible to compare isolates and discover whether a given profile has been seen before, and in what context. For MLST, an excellent website exists (http://pubmlst.org/) with updated databases on a range of microorganisms. This exemplifies the successful use of a library typing method, where the sole sequence can be compared to well-known, validated MLST sequences and a type can be assigned. The GENE network concentrated on exploring the use of the RiboPrinter technology (http://www.ewi.med.uu.nl/gene/), as used for database building, as this equipment is highly standardised in itself. As already mentioned, the PulseNet USA database was the first database based on PFGE profiles of different foodborne pathogens (http://www.cdc.gov/pulsenet/), and was followed by similar networks in Canada (http://www.cdc.gov/pulsenet/participants_pages/pulsenet_canada.htm), Latin America (http://www.panalimentos.org/pulsenet/), and Europe (http://www.pulsenet-europe.org/) [283]. Recently, a similar initiative was developed in Japan [284]. Other PFGE networks have been developed, e.g., SalmGene, encompassing PFGE profiles of *Salmonella* species (http://www.hpa-bioinformatics.org.uk/bionumerics/salm_gene/), HARMONY (http://www.harmony-microbe.net/index.htm), including a range of typing methods for *S. aureus*, with special attention given to methicillin-resistant *S. aureus* (MRSA) isolates, and Listernet, including both PFGE and antibiogram profiles of *L. monocytogenes* (http://www.eurosurveillance.org/em/v10n10/1010-225.asp). Such databases require an extremely high level of standardisation, simple protocols, educated personnel, and continued quality control, to ensure that the data can be trusted. These are a few examples of international cooperation in developing databases for isolate comparison, as other databases are being developed.

The use of typing has been extremely valuable in tracing foodborne outbreaks and pointing out reservoirs; some of the above databases are excellent examples of this.

TRANSLATING TYPING RESULTS INTO CLINICALLY USEFUL INFORMATION AND APPLICATION FOR INFECTION CONTROL

Translating typing results into clinical practice is one of the most important endpoints of a typing exercise. Performing 'real-time' typing may now be feasible in the microbiology laboratory, but once indistinguishable isolates are identified, appropriate clinical action must be taken. Prevention of infection should be the main goal, although in several settings, even the prevention of colonisation (and its spread) is important. In the case of anti-MRSA policies in the northern European countries with low incidence rates, adequate typing plays an important role. The results of typing isolates of (unexpected) MRSA strains should guide the clinical response; for example, in the case of two genotypically indiscriminate isolates of MRSA originating from a single ward or department in Dutch hospitals, the ward or department will be closed. This implies that affected patients will be cohorted, all exposed patients and personnel will be screened for MRSA carriage (and treated when found to be positive), operations will be rescheduled or postponed, and a broad variety of hygiene measures will be implemented. It is obvious that this strategy of 'search and destroy' is costly, and typing data need to be timely and accurate. In the case of closure of intensive care units, false typing results also have very expensive consequences. Ongoing quality assessment of method performance will ensure that results remain reliable. Standardised protocols, training of personnel and detailed inventories of reagents are all absolute prerequisites.

Reports of typing results represent an important diagnostic and didactic tool for clinicians, including infection control staff and those involved in direct patient care. They should be written in an immediately understandable format that both results from, and fosters, further interdisciplinary collaboration. These reports have to include typing and other data concerning isolates, enabling interpretation of results in the light of epidemiology (Fig. 9).

Typing should serve to identify clusters of infection in real time. The first indication of identical types should elicit alarm and lead to clinical action. However, in order to get the best out of typing, some prerequisites must be met even before typing is undertaken. A clear working

Unit Prevention and Control Hospital Infections

Report : Typing of *Acinetobacter* sp.

Date : 2-Aug-06

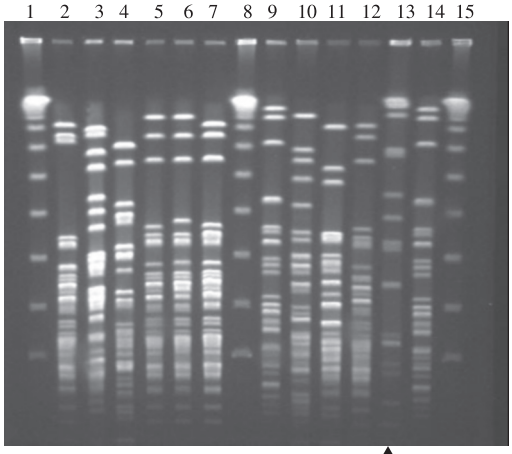
Request from : Hospital A, city B

Investigation done by : Name labworker

Question: Comparison recent isolate (LUH 11629)with previous isolates same department from ICU

Investigation: PFGE with ApaI as restriction-enzyme

Laan 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



Conditions:

1% Seakem Gold agarose

5-20 sec 19 hr

					PFGE 010806
Lane	Strain	Specimen code	Patient name	Specimen date	Rel. pattern
1	marker				
2	LUH 9577	123456-2	Green		
3	LUH 9715	etc	Red		
4	LUH 9758		Orange		
5	LUH 9869		Purple		
6	LUH 10600		Black		
7	LUH 10641		Grey		
8	marker				
9	LUH 8425	reference			
10	LUH 10761		Yellow		
11	LUH 10765		White		
12	LUH 11117		Brown		
13	LUH 11629		Blue		Unique
14	LUH 8425	reference			
15	marker				

Comments:Strain LUH 8425 is clearly different from previious isolates from the same ward.

Signed: ..... *2 August 2006*

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Figure 9. An example of a result report. The report starts with a summary of logistic information, and then the hard laboratory data are shown, usually as gel pictures or plain DNA sequences, followed by a sample identification and some form of data interpretation. This section may be complemented by a tree visualising the interrelatedness among the strains isolated. Finally, concluding remarks are given and the form is authorised by the laboratory head, either a medical or a molecular microbiologist.

hypothesis, or model, must have been formulated, on the basis of available clinical and epidemiological data (e.g., for an outbreak, putative transmission routes and/or source(s). The hypothesis will then be tested by typing; it will also guide the choice of typing method, since different questions may require answers with different levels of reproducibility and/or discrimination. For typing to contribute to infection control, all parties involved (e.g., clinical and laboratory doctors and nurses) must be informed of what will be required of them (from sampling to performing the actual typing), and what consequences the results will have for their practice (e.g., in case one or more

personnel are colonised with the outbreak strain). Similarly, feedback after typing is essential and must include all those involved, not only to translate the results into practice, but also to guarantee continued motivation (Fig. 10). One of the most impressive published descriptions of the benefits, including financial, of molecular typing for infection control outlined a scheme that relied on two simple actions [285]: first, the introduction of REA as a typing method in the clinical microbiology laboratory of a university hospital; and, second, weekly, 45-min meetings of everybody involved in infection control within this hospital. While the method may not have been everyone's immediate first choice, the continuing feedback and the clear-cut aims guaranteed by the weekly meetings made this scheme a success, leading to a 23% reduction in infection rate, and consequent annual savings of approximately $2 000 000. Whether this consequence was fully dependent on the typing itself, or whether increased awareness due to frequent discussions of infection control also played a decisive role, is not really important; it is the net effect that remains important.

To summarise, collaboration at all stages of a typing exercise, clear aims and working hypotheses before typing is begun, reliable quality-controlled data and adequate reporting and feedback all contribute to the total value of typing in clinical practice.

TYPING NETWORKS AND QUALITY CONTROL

A variety of scientific initiatives have led to the establishment of typing networks, some of which have already been mentioned. Several European scientists have made efforts towards standardisation of typing technologies through the ESGEM network (http://www.escmid.org). PFGE has formed the basis for the development of several of the international typing networks including the HARMONY/MRSA effort [203], and, of course, the PulseNet network, initiated in 1996 [286]. Initially started in the USA, and now including PulseNet Europe and other international offshoots, it developed into one of the major typing networks in operation worldwide to date. The initial aim of PulseNet was to develop subtyping synchronisation for food-related pathogens. It started off with *E. coli* O157:H7, several non-typhoidal *Salmonella* serotypes, *L. monocytogenes* and *Shigella* [286]. Currently, *Campylobacter* has been added to the list, which will expand further

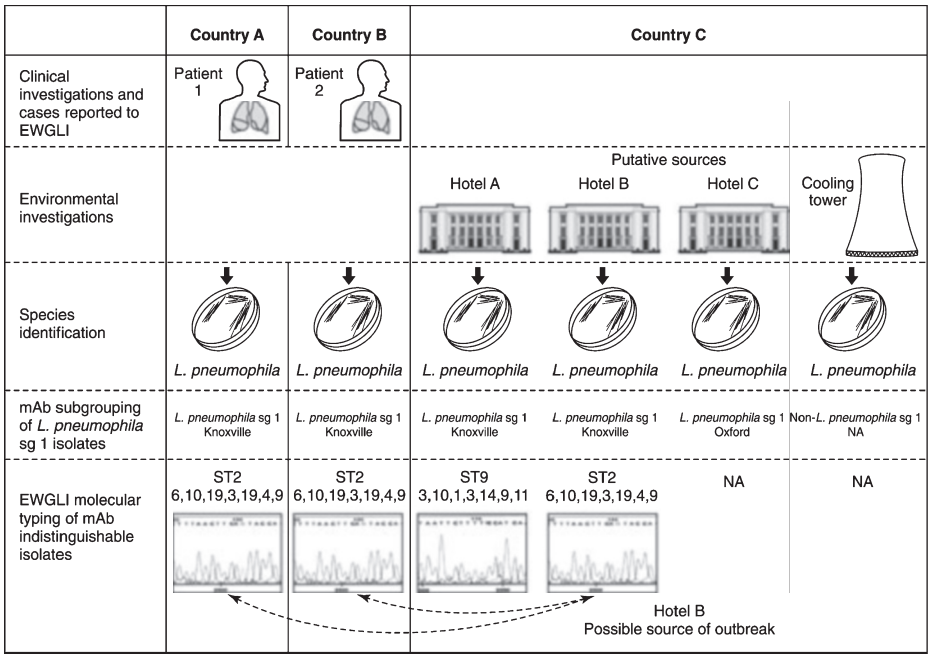

Figure 10. Source tracking for *Legionella pneumophila* according to EWGLI. In cases where patients are identified from remote regions and environmental investigations have been concluded, species will be typed using serogroup-specific antibodies. In cases of identical serogroup and monoclonal subgroup, multilocus sequence typing will be used to further subtype the strains, after which potential epidemiological associations can be derived.

in the foreseeable future [287]. For most of the species currently studied, several thousands of PFGE fingerprints are stored and regularly analysed in a cumulative fashion. It goes without saying that PulseNet relies on extensive standardisation in order to enable fingerprint exchange among centres and centralised computerised analysis. Standardisation involved the development of a universal PFGE fragment size marker standard [288], refinement of the interpretation guidelines [289] and, of course, the development of robust experimental protocols [290]. These efforts are also controlled through annual accreditation of specific, named laboratory personnel, rather than entire laboratories, which has contributed hugely to the current success of PulseNet.

However, there is currently no single specialised institution that focuses on the development of typing standards, both for protocols and for data interpretation. In molecular diagnostics, such initiatives are far more advanced, and molecular typists should profit from the experience gained in this sector. Novel initiatives such as the external quality control assessment scheme developed in Belgium [101] are urgently required.

MOLECULAR TYPING STRATEGIES IN A NUTSHELL

Typing can be useful at different levels: (i) locally, at hospitals or other health institutions; (ii) regionally and nationally, in reference laboratories and research centres; and (iii) globally, through dedicated networks. The choice of methods and the concurrent quality assessment depend on the level at which typing is done.

Local typing

Local typing in clinical microbiology laboratories is undertaken mainly to assess whether an increase in occurrence of particular organisms is due to the spread of a single strain. Currently, the most obvious methods for local typing are PCR fingerprinting and PFGE, and to a lesser extent AFLP, but it is likely that sequence-based methods will soon be more widely applicable at this level too. The choice of typing method will be guided by convenience criteria and will depend on the most common healthcare-associated pathogens ('alert organisms') to be studied.

For these species, collections of unrelated control strains and sets of isolates assumed to be epidemiologically related, together with additional data such as antibiogram and biotype, should be set up. They will then be used to assess precise test conditions for each species to be typed. It is therefore advisable that standardised protocols and qualified advice from specialists in the field are sought at the start. For each typing exercise it is useful to include at least three to five unrelated strains of the same species to confirm discriminatory capacity, as well as a set of related isolates from a previous confirmed outbreak, to assess epidemiological concordance.

PCR fingerprinting

The most simple and rapid genotypic method for local application is PCR fingerprinting. PCR amplification and separation of fragments can be done in 1 or 2 days. For most organisms, crude DNA can be obtained by simply boiling a colony in lysis solution. Every fifth or sixth lane should include a reference sample for normalisation. This sample must be carefully selected and should contain fragments covering the size range of the fragments in the samples. Profiles should, preferably, be judged visually. Computer-assisted analysis of PCR profiles can also be done, and for this purpose the Pearson product moment correlation coefficient (which takes into account band intensity) should be used, and clustering by the unweighted pair-group method using arithmetic averages (UPGMA) is the recommended distance measure. Alternatively, Ward's clustering algorithm could be used. In the case of small numbers of samples on one gel, visual analysis is preferable. In case of doubt about inter-isolate relatedness, highly similar samples should be re-run in adjacent lanes to assess whether they are indistinguishable. Choice of primers, PCR amplification and electrophoresis conditions are important and depend on the micro-organism under investigation. Widely used primers, such as REP or ERIC, are of enterobacterial origin and may not be truly universal. They are, therefore, not ideally suited to all species.

PCR typing is notorious for its susceptibility to minor variations in experimental conditions and reagents, and results may differ among runs, even in one laboratory. Therefore, the method is only suited for comparison of small numbers of samples processed simultaneously and run on one gel. For longitudinal comparison, where large numbers of samples have to be compared over time, this method is not suitable.

PFGE

Today, for most organisms, protocols exist that provide results in 2–3 days. As with PCR fingerprinting, the protocols, although generally similar, are organism-dependent. In electrophoresis, the use of reference samples is essential, as it is for PCR (see above). The buffers and reagents for lysis of cells in the agarose blocks, the enzymes used for DNA digestion, and the electrophoresis conditions are all important. For several alert organisms, well-established protocols are available. If a laboratory is confronted with another organism for which, so far, no PFGE analysis has been done, apart from seeking a protocol for a (closely) related species in the literature, advice can be sought from specialists in the field. For comparison of a few isolates, visual analysis of one gel is easy. 'Fingerprints' can also be analysed by computer-assisted cluster analysis, usually with the band-based Dice coefficient. For local surveillance, it is feasible and worthwhile to set up a database of fingerprints for alert organisms. Every fifth or sixth lane should include a reference sample for normalisation. This sample must be carefully selected and should contain fragments covering the size range of the fragments included in the analysis.

Sequence-based methods

When the appropriate target sequences have been selected, sequence methods, whether they target single or multiple loci (SLST and MLST), are technically simple. After a selective amplification of (part of) the target, the amplified product is sequenced using commercially available technology.

Sequenced mixtures are then read using tools available in the laboratory, which may vary from 'old-fashioned' radioactive slab gels to high-throughput 96-capillary automated sequencers. When sequences have been read, comparative assessment can be undertaken, again using a variety of software tools. Sequence-based methods do not need reference samples, but they do need strict quality control in the sense that the sequence output must be compared with the experimental data for correctness.

Other methods

Implementation and management criteria for the other methods listed in previous sections of this article strongly overlap with those listed above.

THE INTERFACE OF TYPING AND BASIC SCIENCE

Although the interrelatedness between basic microbiological science and bacterial typing is not the main topic of this publication, the association between the two is too important to be completely ignored. The study of bacterial pathogenicity and ecology will continue to profit extensively from strain comparisons at the phenotypic or genotypic level. This has already resulted in a wealth of information on specific virulence genes, their variability and epidemiology, and their involvement in the infectious disease process. A number of fundamental science disciplines such as population biology interact with typing extensively. Among others, studies of the spread and diversity of types and genes in the population, and the selective pressures exerted on that diversity, have contributed to the understanding that clonal and panmictic species exist and thus enriched our understanding of infectious disease epidemiology. An important recent development is the adoption of a systems approach to biological studies (Fig. 11). The aim of this approach is to generate a comprehensive picture of not just the activity of a single gene under a fixed condition, but of complex interactions among multiple genes in an organism, and within a specific environment. Finally, on the technical side, many novel miniature and

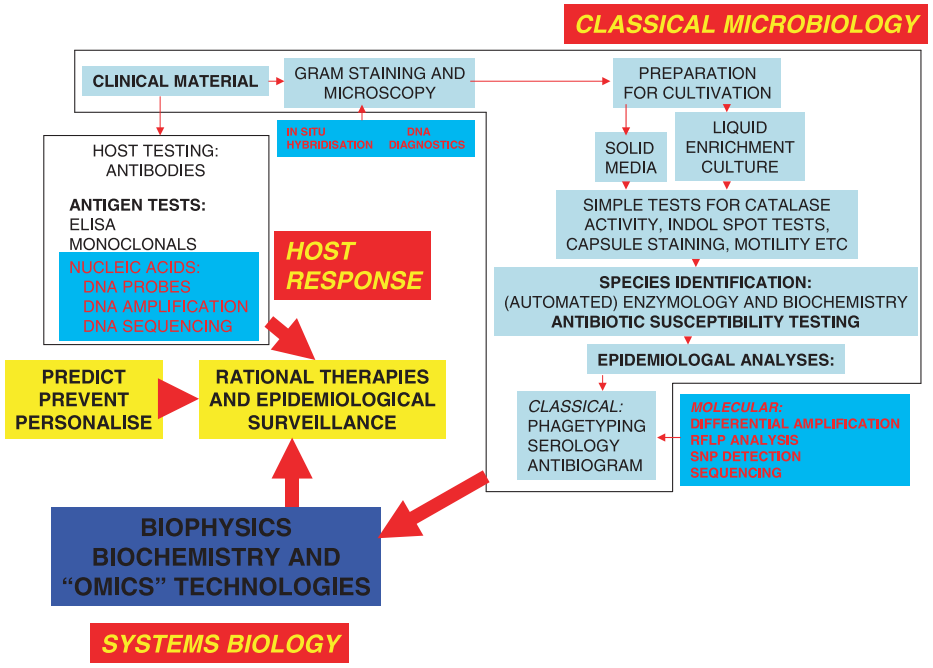

Figure 11. A general scheme showing the position of molecular typing technology in today's microbiology laboratory and the systems biology laboratory of the future. Pale blue: the classic microbiological core technology. Blue: the recent possibilities facilitated by the introduction of molecular technology. Yellow: the integrated systems biology approach. Dark blue: the place of future personalised medical practice. Host-response based diagnosis is indicated where appropriate.

high-throughput technologies have been developed recently, and these will certainly contribute to typing in the not too distant future [291].

AN EXAMPLE OF THE INTERRELATEDNESS OF MOLECULAR TYPING (*Campylobacter jejuni* and its infectious pathology)

The Guillain–Barré Syndrome (GBS) is a post-infectious neuropathy, with the majority of cases resulting from molecular mimicry between human gangliosides and *C. jejuni* lipooligosaccharides (LOS). This was initially shown to be correlated with O-serotypes of *C. jejuni* [292,293]. At a later stage, the molecular mimicry hypothesis was refined on the basis of biophysical and serological studies of the campylobacter LOS (see Koga *et al*. [294] and Moran *et al*. [295] for a review). However, recent epidemiological studies on the LOS composition of larger numbers of GBS-associated strains of *C. jejuni* definitely linked specific LOS genes to this mimicry phenomenon [296,297]. They revealed that certain classes of LOS-encoding gene complexes were clearly associated with GBS-disease-invoking potential. Bacterial typing has thus been instrumental in elucidating the pathogenic process in GBS patients.

A variety of genome sequences has been determined within the genus *Campylobacter*, including two for the species *C. jejuni* [298]. On the basis of these genome sequences, an entire genome array has been developed by different groups (e.g., [299]). An array based on the *C. jejuni* 11168 genome sequence was used to confirm that the overall genome plasticity among *C. jejuni* strains was relatively low [300]. This approach resulted in the identification of several specific loci in the *C. jejuni* genome that showed enhanced evolutionary mutation rates, rendering them suitable for epidemiological studies.

Experimental validation of an extended array (including the RM1221 genome sequence data) confirmed the results of the previous study and helped to define the levels of reproducibility of this method of comparative genomics [301]. A US group also developed

an array system that was primarily used for defining transcription profiles [302,303]. However, this array could also be used to identify genes unique to certain *C. jejuni* isolates [304]. This establishes further levels of *Campylobacter* diversity, and differentially occurring genes or gene segments can be used to develop binary typing approaches. A third array was used to perform extensive phylogenomics and to try and associate type with source of infection [305]. It was demonstrated that new reservoirs for *C. jejuni* can still be identified and that comparative phylogeneomics is one of the methods of choice when trying to define precise population structures. It has to be emphasised that the array technology at the full genome level is not yet suited for day-to-day clinical application. It is, however, obvious that the output of array experiments is highly information-dense and can be used to define inter-isolate identity at the highest possible level and that experimental data will identify novel targets for the development of more dedicated typing systems.

CONCLUDING REMARKS AND PERSPECTIVES

Although typing protocols and networks have come a long way and are increasingly proving their value in the context of infection control and international infectious disease surveillance, there are several areas where future work would be beneficial. Analysis of the molecular events leading to genomic polymorphism in natural and experimental conditions should be undertaken to increase the understanding of the evolutionary mechanisms of bacterial clones as they spread in human populations [26,157]. Collections of extensively typed bacterial pathogens should be assembled and made available via public culture collections [20,21,306]. Dedicated working groups should cooperate in optimising inter-laboratory standardisation and ongoing and independent quality control of genomic typing methods for specific pathogens [25]. Nomenclature used within the various disciplines employing typing technologies should be standardised as much as possible, and should be extended into the field of viral typing [307,308], fungal typing [309], typing of parasites [310], and perhaps even human genotyping. Typing technologies should be made available to those working in areas where economic constraints currently prevent adequate implementation. Appropriate training facilities should be provided where needed and certification could be emphasised. In the end, these initiatives will lead to the establishment of reference standard protocols amenable to multicentre application.

For several organism–method combinations, this stage has already been reached and several such combinations are listed in Table 1. However, there are still many challenges that lie ahead. Significant funding and continuing support are required to sustain existing libraries and develop new methods with the objective of superior and/or more cost-effective approaches.

We have not discussed the medico-legal implications of some typing efforts, and nor have we discussed applications to disease or colonisation susceptibility of the human host. The linkage of typing with disease manifestation has been touched upon only superficially, but surely deserves our undivided future attention. It is currently clear that banding pattern-based methods are in decline and that more transportable, objective and technically simple sequence-based typing systems will be employed in the future and may constitute a new reference standard. Although valuable information may be lost by choosing pure sequence-based approaches, enlarging the number of sequencing targets per strain will, in the end, generate sufficient amounts of data to allow confident deductions on inter-strain relatedness to be made. Reports describing the assessment of hundreds of sequences per organism have been used to generate a phylogenetic tree [311]. Such reports show that, on the basis of pure sequence data, both taxonomically and epidemiologically significant nucleotide variation can be monitored. Typing should be staged carefully and, prior to using typing in the control of infectious disease, dissemination of a set of practical guidelines should be considered and put into practice (see below).

Table 1. Frequently used bacterial genotyping methods: a literature-based survey of multi-centre validated molecular methods and protocols (pubmed screen March 2007)

Pathogens	Hybridisation mediated typing technology	PFGE	MLVA	Single locus sequencing	Multilocus sequencing
Human Bacterial Pathogens					
Acinetobacter baumannii	Brisse S, Milatovic D, Fluit AC, et al. Molecular surveillance of European quinolone-resistant clinical isolates of Pseudomonas aeruginosa and Acinetobacter spp. using automated ribotyping. J Clin Microbiol. 2000;38:3636-45.	Seifert H, Dolzani L, Bressan R, et al. Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of Acinetobacter baumannii. J Clin Microbiol. 2005;43:4328-35.		Yamamoto S, Bouvet PJ, Harayama S. Phylogenetic structures of the genus Acinetobacter based on gyrB sequences: comparison with the grouping by DNA-DNA hybridization. Int J Syst Bacteriol. 1999;49:87-95.	Bartual SG, Seifert H, Hippler C, et al. Development of a multilocus sequence typing scheme for characterization of clinical isolates of Acinetobacter baumannii. J Clin Microbiol. 2005;43:4382-90.
Clostridium difficile			van den Berg RJ, Schaap I, Templeton KE, et al. Typing and Subtyping of Clostridium difficile Isolates by Using Multiple-Locus Variable-Number Tandem-Repeat Analysis. J Clin Microbiol. 2007;45:1024-8.		Lemee L, Dhalluin A, Pestel-Caron M, et al. Multilocus sequence typing analysis of human and animal Clostridium difficile isolates of various toxigenic types. J Clin Microbiol. 2004;42:2609-17.
Coagulase negative staphylococci					Wang XM, Noble L, Kreiswirth BN, et al. Evaluation of a multilocus sequence typing system for Staphylococcus epidermidis. J Med Microbiol. 2003;52:989-98.
Enterococci	Brisse S, Fussing V, Ridwan B,et al. Automated ribotyping of vancomycin-resistant Enterococcus faecium isolates. J Clin Microbiol. 2002;40:1977-84.		Titze-de-Almeida R, Willems RJ, Top J, et al. Multilocus variable-number tandem-repeat polymorphism among Brazilian Enterococcus faecalis strains. J Clin Microbiol. 2004;42(10):4879-81.		Ruiz-Garbajosa P, Bonten MJ, Robinson DA,et al. Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol. 2006;44:2220-8.
Haemophilus influenzae			Schouls LM, van der Ende A, van de Pol I, et al. Increase in genetic diversity of Haemophilus influenzae serotype b (Hib) strains after introduction of Hib vaccination in The Netherlands. J Clin Microbiol. 2005;43:2741-9.		Meats E, Feil EJ, Stringer S, et al. Characterization of encapsulated and non-capsulated Haemophilus influenzae and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol. 2003;41:1623-36.
Klebsiella spp	Brisse S, Issenhuth-Jeanjean S, Grimont PA. Molecular serotyping of Klebsiella species isolates by restriction of the amplified capsular antigen gene cluster. J Clin Microbiol. 2004;42:3388-98				Dranourt L, Passet V, Verhoef J, et al. Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. J Clin Microbiol. 2005;43:4178-82.
Mycobacterium tuberculosis	van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick TM, et al. Related Articles, Links Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993 Feb;31(2):406-9.		Oelemann MC, Diel R, Vatin V, et al. Assessment of an optimized mycobacterial interspersed repetitive- unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. J Clin Microbiol. 2007;45:691-7.		
Pseudomonas aeruginosa	Sarwari A, Hasan R, Lim CB, et al. PCR identification and automated ribotyping of Pseudomonas aeruginosa clinical isolates from intensive care patients. Scand J Infect Dis. 2004;36:342-9.				Curran B, Jonas D, Grundmann H, et al. Development of a multilocus sequence typing scheme for the opportunistic pathogen Pseudomonas aeruginosa. J Clin Microbiol. 2004;42:5644-9.

Table 1. Continued

Pathogens	Hybridisation mediated typing technology	PFGE	MLVA	Single locus sequencing	Multilocus sequencing
Staphylococcus aureus	Lindsay JA, Moore CE, Day NP ET AL. Microarrays reveal that each of the ten dominant lineages of Staphylococcus aureus has a unique combination of surface-associated and regulatory genes. J Bacteriol. 2006;188:669-76.	Murchan S, Kaufmann ME, Deplano A, et al. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant Staphylococcus aureus: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J Clin Microbiol. 2003;41(4):1574-85.	Malachowa N, Sabat A, Gniadkowski M, et al Comparison of multiple-locus variable-number tandem-repeat analysis with pulsed-field gel electrophoresis, spa typing, and multilocus sequence typing for clonal characterization of Staphylococcus aureus isolates. J Clin Microbiol. 2005;43:3095-100.	Aires-de-Sousa M, Boye K, de Lencastre H, Deplano A, Enright MC, Etienne J, Friedrich A, Harmsen D, Holmes A, Huijsdens XW, Kearns AM, Mellmann A, Meugnier H, Rasheed JK, Spalburg E, Stromm-enger B, Struelens MJ, Tenover FC, Thomas J, Vogel U, Westh H, Xu J, Witte W. Related Articles, Links High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J Clin Microbiol. 2006 Feb;44(2):619-21. G	Enright MC, Day NP, Davies CE, et al. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. J Clin Microbiol. 2000;38:1008-15.
Streptococcus pneumoniae					Hanage WP, Kaijalainen T, Herva E, et al. Using multilocus sequence data to define the pneumococcus. J Bacteriol. 2005;187:6223-30.
Streptococcus pyogenes				Neal S, Beall B, Ekelund K, Henriques-Normark B, Jasir A, Johnson D, Kaplan E, Lovgren M, Reinert RR, Efstratiou A. Related Articles, Links International Quality Assurance Study for Characterization of Streptococcus pyogenes. J Clin Microbiol. 2007 Apr;45(4):1175-9.	Enright MC, Spratt BG, Kalia A, et al. Multilocus sequence typing of Streptococcus pyogenes and the relationships between emm type and clone. Infect Immun. 2001;69:2416-27.
Vibrio cholerae		Ader KL, Luey CK, Bird M, Terajima J, Nair GB, Kam KM, Arakawa E, Safa A, Cheung DT, Law CP, Watanabe H, Kubota K, Swaminathan B, Ribot EM. Related Articles, Links Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of Vibrio cholerae. Foodborne Pathog Dis. 2006 Spring;3(1):51-8.			
Food Associated Pathogens					
Campylobacter jejuni		Ribot EM, Fitzgerald C, Kubota K, Swaminathan B, Barrett TJ. Related Articles, Links Rapid pulsed-field gel electrophoresis protocol for subtyping of Campylobacter jejuni. J Clin Microbiol. 2001 May;39(5):1889-94.		Djordjevic SP, Unicomb LE, Adamson PJ, Mickan L, Rios R; Australian Campylobacter Subtyp-ing Study Group. Related Articles, Links Clonal complexes of Campylobacter jejuni identified by multilocus sequence typing are reliably predicted by restriction fragment length polymorphism analyses of the flaA gene. J Clin Microbiol. 2007 Jan;45(1):102-8.	Dingle KE, Colles FM, Wareing DR, Ure R, Fox AJ, Bolton FE, Bootsma HJ, Willems RJ, Urwin R, Maiden MC. Multilocus sequence typing system for Campylobacter jejuni. J Clin Microbiol. 2001;39:14-23.
Escherichia coli		Gerner-Smidt P, Scheutz F. Standardized pulsed-field gel electrophoresis of Shiga toxin-producing Escherichia coli: the PulseNet Europe Feasibility Study. Foodborne Pathog Dis. 2006;3:74-80.	Hyytia-Trees E, Smole SC, Fields PA, Swaminathan B, Ribot EM. Second generation subtyping: a proposed PulseNet protocol for multiple-locus variable-number tandem repeat analysis of Shiga toxin-producing Escherichia coli O157 (STEC O157). Foodborne Pathog Dis. 2006;3:118-31.		Noller AC, McEllistrem MC, Stine OC, Morris JG Jr, Boxrud DJ, Dixon B, Harrison LH. Multilocus sequence typing reveals a lack of diversity among Escherichia coli O157:H7 isolates that are distinct by pulsed-field gel electrophoresis. J Clin Microbiol. 2003;41:675-9.

Table 1. Continued

Pathogens	Hybridisation mediated typing technology	PFGE	MLVA	Single locus sequencing	Multilocus sequencing
Listeria monocytogenes	Lukinmaa S, Aarnisalo K, Suihko ML, Siitonen A. Diversity of Listeria monocytogenes isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. Clin Microbiol Infect. 2004;10:562-8.	Martin P, Jacquet C, Goulet V, Vaillant V, De Valk H; Participants in the PulseNet Europe Feasibility Study. Related Articles, Links Pulsed-field gel electrophoresis of Listeria monocytogenes strains: the PulseNet Europe Feasibility Study. Foodborne Pathog Dis. 2006 Fall;3(3):303-8.			Salcedo C, Arreaza L, Alcala B, de la Fuente L, Vazquez JA. Development of a multilocus sequence typing method for analysis of Listeria monocytogenes clones. J Clin Microbiol. 2003;41:757-62.
Salmonella spp	Clark CG, Kruk TM, Bryden L, Hirvi Y, Ahmed R, Rodgers FG. Subtyping of Salmonella enterica serotype enteritidis strains by manual and automated PstI-SphI ribotyping. J Clin Microbiol. 2003;41:27-33.	pta A, Fontana J, Crowe C, Bolstorff B, Stout A, Van Duyne S, Hoekstra MP, Whichard JM, Barrett TJ, Angulo FJ; The National Antimicrobial Resistance Monitoring System PulseNet Working Group. Related Articles, Links Emergence of multidrug-resistant Salmonella enterica serotype Newport infections resistant to expanded-spectrum cephalosporins in the United States. J Infect Dis. 2003 Dec 1;188(11):1707-16.	Lindstedt BA, Torpdahl M, Nielsen EM, Vardund T, Aas L, Kapperud G. Harmonization of the multiple-locus variable-number tandem repeat analysis method between Denmark and Norway for typing Salmonella Typhimurium isolates and closer examination of the VNTR loci. J Appl Microbiol. 2007;102(3):728-35.		Kotetishvili M, Stine OC, Kreger A, Morris JG Jr, Sulakvelidze A. Multilocus sequence typing for characterization of clinical and environmental salmonella strains. J Clin Microbiol. 2002;40:1626-35.
Organisms with bioterrorism potential					
Bacillus anthracis			Van Ert MN, Easterday WR, Simonson TS, U'Ren JM, Pearson T, Kenefic LJ, Busch JD, Huynh LY, Dukerich M, Trim CB, Beaudry J, Welty-Bernard A, Read T, Fraser CM, Ravel J, Keim P. Strain-specific single-nucleotide polymorphism assays for the Bacillus anthracis Ames strain. J Clin Microbiol. 2007;45:47-53.		Kim K, Cheon E, Wheeler KE, Youn Y, Leighton TJ, Park C, Kim W, Chung SI. Determination of the most closely related bacillus isolates to Bacillus anthracis by multilocus sequence typing. Yale J Biol Med. 2005;78:1-14.
Brucella melitensis			Garcia-Yoldi D, Le Fleche P, Marin CM, De Miguel MJ, Munoz PM, Vergnaud G, Lopez-Goni I. Assessment of genetic stability of Brucella melitensis Rev 1 vaccine strain by multiple-locus variable-number tandem repeat analysis. Vaccine. 2006 Oct 5;		
Fransicella tularensis	Grif K, Dierich MP, Much P, Hofer E, Allerberger F. Identifying and subtyping species of dangerous pathogens by automated ribotyping. Diagn Microbiol Infect Dis. 2003;47:313-20.		Farlow J, Wagner DM, Dukerich M, Stanley M, Chu M, Kubota K, Petersen J, Keim P. Francisella tularensis in the United States. Emerg Infect Dis. 2005;11:1835-41.		

Note to the table: The list was developed based on PubMed searches (March 2007) in combination with personal experience of the authors. While not all references have necessarily addressed all criteria detailed in the guidlines, they have done so to a greater degree than publications not included. We selected papers with a preference for more recent publications. In addition, organisms for which no standardised protocols were available were excluded from the table. Obviously, similar lists can be made for plant pathogens, veterinary pathogens etc. We apologise for omissions and wrong choices: some technologies have been developed to excellence by various investigators and that is where we had to make difficult choices. References listed in this table are not systematically included in the reference list.

THE SEVEN PILLARS OF WISE TYPING

Formulation of test hypotheses
Informed choice of method & control strains
Use of standardised protocols
Careful interpretation of results
Database maintenance
Feedback to all involved
Continuous training and quality assessment

In conclusion, this position paper has endeavoured to sketch the current state of affairs in the field of molecular typing of bacteria. In the process, we have had to make some more or less bold choices and, although we hope that these guidelines will contribute to a fruitful discussion and a rapprochement of all involved in this thriving field, we would like to end by stating that we should be ready and willing to face many more challenges in the near future. In addition, it would be much appreciated if funding agencies would remain open to support of the field.

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