Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems

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Conventional typing methods have been and still are the mainstay in descriptive bacterial epidemiology. To name a few, bacteriophage typing of *Staphylococcus aureus* and *Listeria monocytogenes*, serotyping of *Salmonella* and *Escherichia coli*, and biotyping of *Enterobacteriaceae* have greatly improved our understanding of the natural history of infections caused by these organisms. Likewise, antibiogram typing has for many years been a first-line method in delineation of nosocomial outbreaks. However, conventional methods are usually only applicable to the organisms for which they have been developed. In addition, there is potential variation of expression of the phenotypic traits. With some exceptions, they are usually not sensitive enough to be used in studies of bacterial population genetics.

The resolving power of epidemiologic typing of microorganisms has been expanded by molecular analysis of microbial DNA [1–6]. In contrast to the majority of conventional typing methods based on species-restricted variation of antigenic, metabolic or other phenotypic determinants, similar strategies of DNA analysis can be applied to any microorganism, thereby in general increasing flexibility and typeability. A clonal reproduction within microbial lineages makes DNA structure, at least at the chromosomal level, a potentially stable and specific marker of microbial transmission. In contrast, DNA polymorphisms are present in the genome of all microbial species when unrelated isolates from divergent lineages are considered.

On the other hand, new typing methods are often applied without critical evaluation of their performance characteristics [7]. They often lack standardization in features such as technical procedures, reference material and quality assurance, as well as in the criteria used for the interpretation of results. Basic terminology, including terms like isolate, strain, type, clone or outbreak, may be used with different meanings by workers in the field, thereby increasing the confusion created by the variation in methodology. Progress towards performance comparison or standardization of typing systems has been achieved for a limited number of bacterial and fungal pathogens only [7–13].

The European Study Group on Epidemiological Markers of the European Society for Clinical Microbiology and Infectious Diseases gathered in 1994 to elaborate consensus guidelines for appropriate use and evaluation of typing systems. Workers from 15 hospital, research or reference laboratories in seven European countries discussed and edited this position paper, which endeavors to define terms as precisely as possible, distinguish the major purposes of microbial typing systems, provide criteria for the evaluation of typing system performance and outline the advantages, limitations and unresolved issues related to the current major methods.

DEFINITIONS

It is proposed to use the following definitions, which have been made essentially consistent with the excellent definitions recently proposed by Tenover and colleagues [14]:

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Epidemiological typing system
A system that is used: (1) for discrimination between epidemiologically unrelated isolates belonging to the same microbial species or taxon, based on phenotypic or genotypic character(s) called 'epidemiologic markers'; (2) for recognition of a close relatedness of isolates derived from the same outbreak or chain of transmission, reflecting the fact that they are recent derivatives of a single ancestor cell (see definition of clone below).

Isolate
A population of microbial cells in pure culture derived from a single colony on an isolation plate and characterized by identification to the species level [14].

Strain
An isolate or group of isolates exhibiting phenotypic and/or genotypic traits which are distinc-tive from those of other isolates of the same species.

Reference strain
A well-characterized strain which is preserved for further study.

Type
A specific pattern, or set of marker scores, displayed by a strain on application of a particular typing system.

Clone, or clonal group of isolates (as applied to epidemiologic studies
A group of isolates descending from a common ancestor as part of a direct chain of replication and transmission from host to host or from the environment to host [15]. It should be noted that the term 'strain', 'index strain' or 'outbreak strain' is often used with this meaning in the context of epidemiologic typing [14].

The clonal relatedness of isolates is manifested by their display of a significantly higher level of similarity of their genotype and/or phenotype than can be expected for randomly occurring and epidemiologically unrelated isolates of the same species. The quantitative threshold of similarity used as a working definition of a clone should be adjusted to the species studied, the typing system(s) used and the time–space frame of the epidemiologic investigation.

This definition in probabilistic terms may be less stringent than definitions of a clone used by microbial geneticists [15–17]. Analysis of bacterial population structure shows that many bacterial species, such as *E. coli* and *Salmonella enterica,* have a clonal structure, but that others, such as *Neisseria gonorrhoeae* are panmictic i.e. interstrain recombination is so frequent that it obscures vertical lineages [18]. A further complexity arises from the fact that some highly virulent clones of pathogenic bacteria may become widespread and remain stable for prolonged periods [11,13,16–19]. Finally, phenotypic markers can be misleading for assessment of clonality because of the possibility of evolutionary convergence. A strong environmental pressure may select the emergence of similar phenotypes in distant lineages through independent events, as illustrated by multiply resistant bacteria causing nosocomial infection.

PURPOSE OF EPIDEMIOLOGIC TYPING

In general terms, epidemiologic typing systems are used to study the population dynamics and the spread of bacteria and other microorganisms that undergo non-sexual (clonal) reproduction, in nature or in the clinical setting, at levels ranging from a single host up to the worldwide population ecosystem.

Specific purposes include the study of bacterial population genetics, the study of the pathogenesis of infection, epidemiologic surveillance of infectious diseases and outbreak investigation.

Study of bacterial population genetics

Some molecular typing systems may be applied to large samples of isolates from various origins in order to determine the intraspecies population structure, and derive phylogenetic hypotheses from this structure [16–18,20]. Such basic knowledge may be useful for practical applications, e.g. for defining the level of similarity for a biologically plausible 'clone' that can be used for outbreak investigation. For example, macro-restriction analysis of the *Pseudomonas aeruginosa* genome indicates that the average genomic pattern similarity of unrelated strains ranges between 20% and 60% with a mode at 35%, whereas clonally derived strains from a single host cluster at similarity levels above 80% [20,21].

Study of pathogenesis and natural history of infection

Typing systems may also be used in clinical studies for delineation of patterns of colonization and for identification of sources of transmission of infecting microorganisms from the endogenous or exogenous microflora. This contribution to an understanding of epidemiology and pathogenesis assists with the development of prevention strategies [22].

Surveillance of infectious diseases

Typing systems may contribute useful information to epidemiologic surveillance of infectious diseases, defined as a systematic, ongoing process of data collection, analysis, interpretation and dissemination aiming at

following up trends in disease frequency and associated risk factors in a target population.

Typing will provide information on microbial type distribution in human populations over time and place (descriptive epidemiology). This can be applied to surveillance programs targeted at the local, regional, national or global level. Of special interest will be the monitoring of markers associated with pathogenicity, immunogenicity or drug resistance, as shown, for example, in surveillance of cholera with the emergence of a new epidemic strain in Asia [23].

In the short term, this application may include periodic analysis for detection of clusters of pathogens with similar type and common time–space origin, to provide 'early warning' of potential outbreaks. In the long term, surveillance assists in the planning of health services and preventive interventions (e.g. vaccine development and immunization programs).

Definitive typing, based on standardized methods that are sometimes referred to as 'library typing systems', where types can be compared over time and place between studies from different laboratories, is required for this application to surveillance of infectious diseases [7].

Outbreak investigation and control

An outbreak can be defined as a temporal increase in the incidence of infectious morbidity in a given population, or, alternatively, as a temporal increase in the frequency of colonization by a given microorganism, with or without a concurrent increase in infectious morbidity. Outbreaks are often associated with an increased rate of transmission of a given pathogen. Colonization without disease may also reflect microbial spread in a population and may be worth investigating (e.g. spread of multiresistant clones as part of the commensal flora of hospitalized patients).

Typing systems are applied primarily to assist epidemiologic studies in testing, and also in generating hypotheses about:

- the extent of epidemic spread of microbial clone(s) in an exposed population;
- the number of clones involved in transmission and infection;
- the identification of the source(s) of contamination and the vehicles of transmission;
- the identification and monitoring of reservoirs of epidemic clone(s) in the population and/or the environment;
- the evaluation of the efficacy of control measures aimed at containing or interrupting the spread of epidemic clone(s).

This application of typing to interventional epidemiology, whether in the hospital setting or the community, requires first-line typing, or screening methods, that are used for initial assessment of microbial isolate relatedness and which provide rapid results at the level of field investigation. Confirmation by secondary typing methods may be obtained when more confidence is needed to design or refine appropriate control measures.

Comparative typing, sometimes referred to as strain fingerprinting, where only a limited set of outbreak-related and unrelated strains are compared for type distribution without reference to microbial types circulating elsewhere, is sufficient for this application to outbreak investigation [7]. Nevertheless, it is still necessary to validate the discriminatory power of the method, at least for microorganisms from patient populations and ecologic niches relevant to the outbreak setting.

CRITERIA FOR EVALUATION OF TYPING SYSTEMS

These criteria can be subdivided into two categories: performance (efficacy) and convenience (efficiency). Because different investigations may require a different level of efficacy and efficiency, there is no ideal typing system for universal use [1].

Performance criteria

The following performance criteria [1] are proposed: typeability, reproducibility, stability, discriminatory power, epidemiologic concordance and typing system concordance. All performance criteria of typing systems should be evaluated for every microbial species and ecosystem under study.

Typeability

The typeability is the proportion of strains that are assigned a type by the typing system. The formula reads:

$$T = \frac{N_t}{N}$$

where: N_t is the number of isolates assigned a type and N the number of isolates tested. For a marker to be useful, T should be as close to 1 as possible.

Reproducibility

The reproducibility is the ability of a typing system to assign the same type to a strain tested on independent, separate assays. The formula reads:

$$R = \frac{N_r}{N}$$

where N_r is the number of isolates assigned the same type on repeat testing and N the number of isolates tested.

For complex marker systems, the differentiation criteria used for type assignment should be taken into account. The reproducibility of marker pattern and that of type assignment may be different and both need to be evaluated.

To evaluate R, it is important to design serial experiments that assess the influence of all technical steps involved in type assignment, including: strain preparation (DNA or protein extraction, growth conditions where relevant); test and reagent batch (intratest, intertest); laboratory (inter-laboratory, same equipment, standardized protocol); observer interpretation and matching of complex patterns (e.g. DNA restriction or amplification patterns) – inter-observer, computerized assignment versus subjective, visual assignment.

Because reproducibility of a method will greatly affect its discriminatory power, R should be ideally > 0.95 for all applications, and even higher for reliable definitive typing. Many typing methods offer sufficient within-test reproducibility for comparative typing of a limited number of strains. However, most molecular typing systems are yet to be shown to be sufficiently reproducible or standardized for use in definitive typing.

For typing systems that do not reach this level of reproducibility, such as phage or bacteriocin typing, technical and biological variation needs to be considered when interpreting differences. Therefore, rules such as two reaction differences can be applied to distinguish types with these systems.

Stability

The stability of epidemiologic markers con-ditions the ability of a typing system to recognize the clonal relatedness of strains derived in vitro or in vivo from a common ancestor strain, despite the phenotypic or genomic variation that may occur during laboratory storage and replication, or during clonal dissemination in nature, especially over prolonged periods or in large-scale epidemics. Because mutations and intra- and intergenomic recombination related to integration or mobilization of plasmid, phage and transposable DNA occur at frequencies depending on species, strain and environmental conditions, the stability of markers tested by every system should be evaluated for every microbial species and ecosystem under study.

The in vitro stability is assessed by comparing strains tested before and after storage for a fixed period of time, and after serial passage on specific culture media. We suggest that at least 10 strains are studied after every fifth passage in an experiment of 50 serial passages (thus yielding a total of 100 tests). The formula reads:

$$S = \frac{N_r}{N}$$

where N_r is the number of tests in which the same strains were correctly assigned the same type on repeat testing and N is the total number of tests.

The in vivo stability can be estimated by comparing strains tested before and after passage in a suitable animal model, or sequential isolates recovered from cases of persistent colonization or infection, from different anatomic sites within the same patient or along the course of epidemiologically well-documented outbreaks. Clonal variants that may be recognized to arise in such settings should be taken into consideration for adjusting the working definition (level of similarity) of a clone as applied to future studies of the organism over similar time–space frames. Such variation must be distinguished from the fortuitous occurrence of different strains in these situations in vivo.

Discriminatory power

The discriminatory power is the average probability that the typing system will assign a different type to two unrelated strains randomly sampled in the microbial population of a given taxon. This probability, as shown by Hunter [24], can be expressed by the formula of Simpson index of diversity, which reads:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} n_j(n_j - 1)$$

where D is the index of discriminatory power, N the number of unrelated strains tested, S the number of different types, and n_j the number of strains belonging to the jth type, assuming that strains will be classified into mutually exclusive categories. Otherwise, if differentiation criteria are used, such as for phage type data, a more general version of this formula is given by the equation

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} a_j$$

where a_j is the number of strains identical to the jth strain [24]. A computer program to calculate D is available from Hunter [24].

D depends on the number of types and on the homogeneity of frequency distribution of strains into types; ideally, each strain should have a different type ($D = 1$). For the purpose of calculation, non-typeable strains can either be excluded or can be grouped together, but this latter approach does not imply that they are of the same type.

For typing systems based on complex marker scores, such as genomic fragment patterns, that are subjected to quantitative analysis, the D value should be calculated at the similarity threshold levels that are used for defining clonal groups.

To comply with the conventional 5% level of acceptable probability of type I error, D should ideally be >0.95 for a typing system to be used as single typing method. Less discriminating typing systems may be used in combination to reach a combined $D > 0.95$.

If the method is not 100% reproducible, the D value should be calculated by interpolation at the 95% reproducibility level, as explained by Hunter [24].

Test population

Special attention should be paid to the appropriate selection of a microbial test population for evaluating the typeability and the discriminatory power of typing systems. A large test population of isolates correctly identified to the species level must be assembled to reflect as much as possible the diversity expected in the species as a whole, or at least in the microbial population to which the typing system will be applied [7–10]. This includes the ecologic niches that may be the subject of future investigation, such as particular patient populations (including age category, immune status, type of hospital, ward, geographic origin), and environmental reservoirs relevant to epidemiology (e.g., zoonosis, waterborne infection). It also deals with specific patterns of infections, because there may be pathovar-restricted genomic diversity at subspecies level [16,17]. The test population should include strains that are presumably unrelated epidemiologically, on the basis of detailed clinical and epidemiologic data. Except for reference strains, the majority of isolates should be of recent origin. Therefore, assessment of type-ability/discriminatory power should be repeated at periodic intervals.

Large size collections of unrelated strains ($N > 100$), not selected on the basis of type characteristics, are recommended for the unbiased and precise comparison of the T and D values of different typing systems [7,8]. For surveillance and population genetic studies, even larger collections of strains will usually be needed [16,17]. Analysis of such large collections remains technically difficult for many molecular typing systems, because it requires highly standardized electrophoresis conditions and computer analysis.

In outbreak investigations, it may be useful to confirm the appropriate level of discrimination of the typing system(s) used by comparing the outbreak-related strains to a set of control strains ($n = 10$ to 30) from a similar time period, locale and patient population, but which are, *a priori*, not epidemiologically related. A significantly different type distribution among outbreak-related and unrelated strains will strengthen the probability of a recent clonal link between the outbreak strains.

Epidemiologic concordance

This is the probability that epidemiologically related strains derived from presum-ably single-clone outbreaks are determined to be similar enough to be classified into the same clones [9,10]. It is in fact a particular application of in vivo stability within a human population rather than in a single or a few hosts. The formula reads:

$$E = \frac{N_e}{N}$$

where N_e is the number of strains assigned to epidemic clones and N the number of strains tested from well-defined outbreaks. It is desirable that several sets, e.g. five or more, of outbreak-related strains ($n = 5$ to 10 per set) are included in the sample of study strains. Ideally, E should be equal to 1.

Typing system concordance

Although epidemiologic relatedness should be the gold standard for clonal delineation and for evaluating typing system specificity, it should be interpreted with caution. For example, multiple clones may be co-transmitted during the course of a single outbreak [26]. Therefore, it is of interest to compare the results of independent typing systems. Isolates that are concordantly grouped into highly similar types by several systems are increasingly more likely to be clonally related [9–11,13,26].

In such comparisons, genomic typing systems may in general be given more weight than phenotypic systems. The latter systems are less directly correlated with clonal descent because of the possibility that they are skewed by variable marker expression and evolutionary convergence. Likewise, typing systems exploring polymorphism at multiple sites of the whole genome are more representative than typing systems exploring variation at a single gene locus.

Convenience criteria

Convenience criteria may be important for the selection of appropriate typing system(s) depending on a number of factors, including the scale of the

epidemiologic investigation, the timeliness of information needed and the financial and technical resources available. The following criteria may be considered: flexibility, rapidity, accessibility and ease of use. The flexibility reflects the range of species, or higher taxonomic groups, typeable with minimal modifications. The rapidity of typing systems varies from same day to several weeks. Many typing methods can provide results within 24 to 72 h, which is sufficiently rapid for most outbreak investigations. Accessibility depends upon the availability and cost of reagents and equipment and the skills required for a given method. The ease of use includes the technical simplicity, the workload, the suitability for processing a large number of strains and the ease of scoring and interpretation of results.

ASSESSMENT OF PHENOTYPIC TYPING SYSTEMS

Multilocus enzyme electrophoresis

Based on the phenotypic analysis of electrophoretic variants of a set of housekeeping enzymes, this scores allelic variation of multiple genes and thus is an indirect but well-validated genotyping method [27]. It may be used as a reference method for defining the phylogenetic structure of clonal lineages in microbial populations [16,17]. However, it is neither a rapid nor a widely accessible system.

Biotyping

This includes a number of biochemical characters that are known to vary within a given taxon. The characters used depend upon the species. The typeability is typically excellent. Discrimination is variable according to the available markers and to the species but is often low, unless a large number of well-selected characters are included in the test scheme. Stability is dependent on species. The method is easy to use, score and interpret, even in small laboratories. It is also technically simple and can be performed at a low cost for large numbers of isolates. If reproducibility is demonstrated, it can be used as a definitive typing method [11,28].

Antibiogram typing

Susceptibility testing to a number of antimicrobial agents, including drugs and chemicals not relevant for treatment, can be performed either with diffusion or dilution methods. Resistance breakpoints that are biologically discriminating for detection of acquired resistance determinants may not coincide with therapeutic breakpoints used in the clinical microbiology laboratory. Moreover, minimal inhibitory concentrations or quantitative analysis of growth inhibition zone size are more informative than qualitative resistance patterns. Squared euclidian distance is a measure suitable for multivariate cluster analysis of antibiogram similarity between strains, adjusted for the level of reproducibility of the system [11,29].

Antibiogram typing can, with a relevant selection of markers, be applied to most microbial species. Discrimination is dependent on the diversity and relative prevalence of detectable acquired resistance mechanisms in study isolates. The stability of resistance pattern can be insufficient for its use as a clonal marker, especially if resistance determinants are plasmid borne or expressed under control of complex regulatory systems [10,11,26,29].

The antibiogram is one of the most valuable first-line typing methods in clinical laboratories. It has the advantages of being easy to use, score and interpret, even in small laboratories. It is a technically simple and low-cost system suitable for testing large numbers of isolates. Good reproducibility allows its use for definitive typing if a standard method and set of marker compounds are adhered to.

Serotyping

Determination of surface antigens by using a defined set of polyclonal or monoclonal antibodies is applicable to single species or genera only. Typeability, frequency of cross-reactions and discrimination of serotyping schemes are variable according to species, pathovar, the number of antigenic determinants scored and the specificity of reagents [1,8,9]. The stability is dependent on the species, but is often good. Serotyping is technically simple and well suited for testing large number of isolates. With adequate reagent and test quality control, it can be a reproducible, definitive typing method of wide applicability. However, only reference laboratories can perform reliable serotyping for some organisms for which properly cross-absorbed polyclonal antisera or standardized monoclonal antibodies are not commercially available.

Protein gel electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole cell proteins, cell envelope proteins or culture supernatants have been applied with varying degrees of success for discrimination of microbial strains [30]. Standardization of preparation and testing conditions is important and can be successfully achieved with inclusion of multiple controls per gel and computer-assisted analysis of electrophoretic patterns [11,30]. Resolution and discrimination can be improved by combining SDS–PAGE with immunologic analysis (immunoblotting) [1,10].

Phage typing and bacteriocin typing

Based on the production of specific lytic patterns of test isolates exposed to a defined set of bacteriophages, or on specific patterns of bacteriocin-mediated lysis of a defined set of susceptible strains, these traditional typing systems are restricted to a limited number of species. Typeability and discrimination are variable according to species and strain but typeability is most often incomplete and should be checked over time, especially when new clones are introduced. The stability is dependent on species and clone but is usually sufficient for outbreak investigation. Phage typing is successfully applied for several major bacterial pathogens, including *S. aureus* and *P. aeruginosa* [9,10,31]. Additional phages may need to be included when untypeable clones show increased prevalence.

The production and continuous quality control of phages is important and time-consuming. Production of international standard phages should be performed only in reference laboratories, which may distribute 'ready-made' phages for use in other laboratories with the relevant control procedures. Large numbers of isolates can be processed readily. Interpretation of results is not easy and requires training and experience. Phage typing can be used as a definitive typing method for long-term surveillance of large patient populations [31].

ASSESSMENT OF GENOMIC TYPING SYSTEMS

Plasmid typing

Determination of the number and size of plasmids by agarose gel electrophoresis enables typing of many bacterial species [2]. Marker identity is preferably based on restriction endonuclease analysis of plasmid DNA. The typeability and discrimination are variable according to the bacterial species [2]. The stability of plasmid content has been found insufficient for use as a clonal marker in some studies [2,10,26]. It is best combined with other genomic typing methods (at the chromosome level) to distinguish between spread of a clone and that of a plasmid [10]. Plasmid typing is of particular importance in studies of drug-resistant clones and should, in some cases, include more detailed genetic mapping and analysis of transposon- and plasmid-borne genes.

Typing by restriction fragment length polymorphism (RFLP) analysis of genomic DNA

Conventional restriction endonuclease analysis (REA) of genomic DNA

In this method, the chromosome is cut with frequently cleaving enzymes into several hundreds of small fragments, which are partly separated by conventional gel electrophoresis into complex patterns [3]. Pattern resolution can be improved by careful enzyme selection, or by using specific separation and labeling conditions (PAGE, silver staining), or by limiting the observation window on the gel [3,25]. REA is a rapid method which is mostly limited to comparative typing purposes [3]. The method is, under standardized conditions, very reproducible and dis-criminatory.

Macrorestriction endonuclease analysis of genomic DNA resolved by pulsed-field gel electrophoresis (PFGE)

The chromosome is cut with low-frequency cleaving enzymes into less than 30 large fragments which are separated into clear patterns by PFGE. This method has shown remarkable discrimination and is a reproducible, widely applicable method for comparative typing of many bacterial species [1,4,10,13,21,22,25,26]. Macro-restriction takes 2 to 4 days to obtain results and relatively expensive PFGE equipment is required. Inter-laboratory standardization has not yet been achieved for use of PFGE as a definitive typing system.

RFLP with nucleic acid probes, including IS fingerprinting and ribotyping

This strategy uses probes (genes, IS elements, rRNA), to hybridize endonuclease restriction digests of genomic DNA separated by conventional electrophoresis and transferred to a membrane (Southern blotting). Ribo-typing is a universally applicable and reproducible method which still requires technical guidelines for optimization and general rules for interpretation [5,8,10,13,29]. The level of discrimination achieved with ribotyping varies according to species, but is typically lower than with REA and macrorestriction [5,10,13]. RFLP with hypervariable probes is restricted to a single species, and requires a great expenditure of time for the search for a suitable locus. It is a reproducible and discriminant typing tool for important pathogens like *P. aeruginosa* [3,8]. IS fingerprinting is well established as a typing method for major pathogens such as *Mycobacterium tuberculosis* [12] and *Staphylococcus aureus* [13], but it also requires extensive search and evaluation of suitable sequences restricted to a single species.

In general, these methods are reproducible and provide good discrimination for some species although they only cover a single or a few genomic loci. Some applications are well standardized, such as *IS6110* typing of *Mycobacterium tuberculosis* [12]. However, they are relatively laborious methods and require several days to provide results.

PCR fingerprinting: arbitrarily primed PCR (AP PCR), randomly amplified polymorphic DNA (RAPD) or inter-repeat element PCR typing (rep-PCR)

These various PCR-based typing techniques use either arbitrary primers, or primer pairs directed outward from repetitive elements for amplification of short spacer sequences lying between repeat motifs in microbial genomes [6].

They are universal typing methods and exhibit a high and easily adjustable level of discrimination [6]. Modified protocols enable detection of polymorphism at the expression (mRNA) level [6].

Major advantages of these techniques include flexibility, technical simplicity, wide availability of equipment and reagents and rapid (same day) turnover. Current critical problems of this approach include: (1) optimization and choice of reagents/protocols; (2) inter-run and inter-laboratory reproducibility; and (3) interpretation of amplimer patterns and differentiation criteria used for clone delineation [6,32]. For these reasons the PCR-fingerprinting methods are not recommended for use in laboratories without extensive knowledge about and experience in both typing and PCR.

INTERPRETATION OF RESULTS OF MOLECULAR TYPING

Theoretically, the ideal method to study the genetic relatedness of bacterial strains at the subspecies level would be the determination of the complete sequence of their genomes [1]. Several less comprehensive but more practical methods are now used to assess polymorphism in microbial genomes, as outlined above. These methods raise questions about interpretation of results, which are often complex to analyze. Criteria for interpretation of genomic polymorphism in epidemiologic studies of microbial pathogens will be different according to the purpose of the study, whether dealing, for example, with local outbreak investigation, or with large-scale surveillance. There are several general issues, however, that are related to quantitative analysis of microbial genomic polymorphism for epidemiologic typing.

Number of loci

It is accepted that, by using multilocus enzyme electrophoresis, about 30 loci need to be scored to provide reliable estimates of subspecies clonal structure, although this number may vary depending on locus diversity [27]. By analogy, we suggest that about 30 genomic sites, roughly equivalent to that number of DNA bands in restriction/amplification patterns, may need to be analyzed to obtain stable and accurate estimates of genomic diversity. The number of restriction enzymes, probes or primer sets may be adjusted accordingly [13,29]. However, it is often more practical and also more informative, to combine two or three independent phenotypic/genomic typing systems than to increase the probe/reagent range of a single genomic typing system [1,10,11,13,19,25,26,29].

Analysis of genomic pattern similarity

Strain relatedness is inferred from genomic typing methods based on DNA size after separation by electrophoretic methods either in terms of absolute number of band differences, or as percentage similarity of banding patterns.

The latter is most commonly calculated as a Dice coefficient, following the formula:

$$S_D = \frac{2a}{2a + b + c}$$

where a is the number of matching fragments and b and c are the numbers of mismatching fragments in each lane of a pairwise comparison.

The absolute number of band differences is a measure that needs to be interpreted with caution: its weight will be proportional to the denominator, that is the number of resolved DNA fragments. This number is related to the choice and number of genomic sites probed, which depends on the number and nature of enzymes or primers used, as well as on the amplification and separation conditions.

The interpretation of number of band differences can be improved by considering the minimum number of genetic events necessary to produce the observed variation, as recently proposed by Tenover et al [14]. Genetic analysis of the molecular event(s) (mutation, deletion, insertion) associated with pattern variation is the preferred approach to measure relatedness, but is not generally feasible [6,33]. As a crude rule, a one- to three-band difference observed between patterns obtained by PFGE, RFLP, or, by analogy, AP PCR typing with a resolution of >10 DNA bands may be equated with ≥ 1 genetic event; a four- to six-band difference may likewise be assigned to ≥ 2 genetic events. The number of genetic events/band differences considered for separating clonal groups depends on several technical and biological factors, including: resolving power of typing system (denominator), reproducibility, genomic plasticity of the organism and the time scale of the study [10,12,13,15,25,26,28,33].

In a very useful guideline paper for outbreak investigations limited to about 30 isolates, Tenover et al. have proposed a stepwise approach to determine strain relatedness on the basis of the likely number of

genetic events associated with PFGE pattern mismatches [14].

Dice coefficients of pattern similarity can be submitted to cluster analysis to produce dendrograms or graphical display of relatedness hierarchy among the strains [22,25,27,28]. Genomic pattern similarity values need to be based on a sufficiently large, and preferably even, number of genomic sites/bands for each strain. Thus, if low and variable copy number RFLP probes (e.g. IS sequences) are used, a composite similarity coefficient needs to be constructed to reach a stable denominator across the sample of strains under study. Using algorithms such as unweighted pair group using mathematical average (UPGMA), the dendrogram topology depends on strain composition of the sample. Therefore, any inferred measure of interstrain relatedness is relative only to the overall relatedness in that particular sample of strains. In addition to dendogram topology, non-parametric tests may be used to test the significance of intra- versus intergroup genomic pattern similarity, providing that groups of strains are defined on the basis of epidemiologic origin [25].

In general, genomic pattern similarity cannot be considered as an exact measure of genetic distance, because band positions are not independent: as mentioned earlier, a single point mutation may introduce as many as three band differences in a DNA restriction pattern, if it is associated with the creation or elimination of a restriction site. However, under certain conditions, genomic macrorestriction pattern can be used as a measure of genetic relatedness at subspecies and species levels [20].

RESEARCH NEEDS

Collections of microbial pathogens characterized by different typing systems displaying type diversity should be assembled and made available by depositing these into public culture collections [7,8,32]. Collaborative groups with expertise in specific pathogen–typing system combinations should be further developed to undertake large scale comparative studies of the performance of typing methods, using collections of both unrelated and epidemic-related strains of various geographic origins [7–12,32].

Working groups should cooperate toward optimization and inter-laboratory standardization of genomic typing systems for specific pathogens, including: DNA preparation, reagents used (e.g. enzymes, probes, primers), equipment and assay protocol, molecular size markers and reference strains, pattern analysis principles, hardware and software used for image digitization, pattern normalization, recognition, labeling and matching [12].

Analysis of molecular events leading to genomic pattern polymorphism in natural and experimental conditions should be conducted to increase the understanding of the evolutionary mechanisms of bacterial clones as they spread in human populations [13,33].

The European Study Group on Epidemiological Markers is an open forum for discussion of these issues and will welcome comments and criticism of the proposed guidelines. It is also promoting the development of collaborative projects addressing the research needs outlined above.

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