Applications of multilocus enzyme electrophoresis in medical microbiology

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Abstract

Multilocus enzyme electrophoresis has been used since the beginning of the eighties for the study of numerous microbial pathogens. The principles of this technique are presented and its application for the study of population genetics, of systematics, and of molecular epidemiology are discussed. Several examples from the work of the author and from other laboratories illustrate the usefulness of the method in these three fields of medical microbiology. Finally, multilocus enzyme electrophoresis is compared with recent methods of molecular biology and the future of multilocus enzyme electrophoresis is discussed. © 1997 Elsevier Science B.V

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1. Introduction

Multilocus enzyme electrophoresis (MEE or MLEE), also called isoenzyme typing, has been used for several decades in the field of eukaryotic population genetics. It was however, only in the beginning of the eighties, that the pioneer work of Selander and collaborators, mainly on *Escherichia coli* and *Shigella* genetics [1–5] has triggered the interest of medical microbiologists for this method. Since then, numerous studies have made use of MEE with invaluable results for knowledge of the natural history of infectious diseases. Results similar to those determined by MEE can also be obtained by more recently developed techniques of molecular biology. However, these techniques still have to be validated extensively and, despite its cumbersome nature, MEE will probably remain a gold standard for population genetics of microorganisms for an extended period of time.

2. Principle of the method

In MEE, a pure culture of the organism to be tested is grown under optimal conditions and the cells are washed and lysed by a suitable method (ultrasound treatment, vortexing with glass beads, or repeated freezing and thawing). The water-soluble fraction of the lysate is recovered by centrifugation. The cytoplasmic enzymes contained in this solution are then separated as functions of their molecular mass, electrical charge and conformation by electrophoresis under non-denaturing conditions. This electrophoresis is usually performed in starch or polyacrylamide gels, but other media such as cellulose acetate or agarose have also been applied for this
The applications of MEE are manifold. They have led and will continue to lead to important findings with both theoretical and practical consequences for our understanding of many microbial pathogens. The present text is not an exhaustive review of all the major discoveries obtained by use of MEE in medical microbiology, but will rather focus on a few examples from our own work and from other laboratories to illustrate the power of MEE for population genetics and molecular epidemiology of infectious diseases.

3. Panmixy and clonality in populations of pathogenic microorganisms

Genetic recombination between organisms of a microbial population can occur in different ways. When it occurs, sexual reproduction is probably the major way of gene exchange in natural populations of protozoa and fungi. In some bacterial species, the main route of horizontal genetic transfer seems to be by transformation. However, in other non-naturally transformable species, recombination is also present (though at lower frequency) and other mechanisms like conjugation and transduction may explain this
phenomenon [10]. Exchange of genetic material as demonstrated by in vitro experiments may be possible in a microorganism, but may be too rare an event under natural conditions to significantly influence long term evolution of the organism. Several statistical techniques have been developed in order to analyse the significance of this phenomenon in natural populations. These tests are of two kinds: segregation tests and recombination tests [11,12]. The former tests are applicable only to diploid organisms, whereas the latter apply to both haploid and diploid organisms. In segregation tests, populations showing a significant bias in the frequency of genotypes at each locus when compared to those expected under Hardy-Weinberg equilibrium (a situation in which random mating and random association of alleles occurs) are considered as possibly clonal. Good illustrations of these techniques have been published by Tibayrenc and coworkers [13] in the field of parasitology. In recombination tests, the recombination between different loci is examined. In the absence of meiosis and recombination, or significant horizontal transfer, the alleles present at different loci are not redistributed regularly or exchanged with other organisms. Thus, in a clonal population, the alleles of different loci show a strong association (linkage disequilibrium). A lack of recombination also leads to the recovery of identical genotypes in geographically or temporally widely separated populations. However, the widespread recovery of a particular genotype may also have other origins and has to be interpreted cautiously [14]. Finally, the lack of recombination between loci in a clonal population also leads to a large congruence of results obtained with several methods based on different markers. In panmictic populations, the independence of the different loci brought by constant recombination leads to a lack of such congruence (for example between MEE and arbitrarily primed PCR results or between MEE and restriction fragment length polymorphism...
results). It is clear that the results of the tests mentioned above depend on many factors, including the sample examined. A well designed strategy for the choice of the population under study (size of sample, geographical origin, distribution in time, origin of isolates in terms of clinical and ecological significance) is crucial for the reliability and meaning of the results and a biased sample may lead to completely inaccurate conclusions.

The assessment of clonality is probably the most important step in the study of a microorganism by population genetic methods and may have important consequences for the approach to be used for the study of its epidemiology. Several situations have been recognized in pathogenic microorganisms by MEE. At the one end of the spectrum, *E. coli* and *Salmonella enterica* or *Candida albicans* have been shown [3,14–17] to be principally clonal microorganisms (without significant recombination and exchange of selectively neutral genetic material). This does not exclude the horizontal transfer of genetic material, but these exchanges do not significantly disrupt the clonal nature of the populations [10]. In such cases, MEE may allow the researcher to draw an evolutionary backbone on which the evolution and transfer of factors of medical importance under strong environmental selective pressure can be studied and followed. Among others, the evolution of flagellin genes in *Salmonella* has been studied in this way, demonstrating the role of horizontal transfer of genetic material as an important factor in the evolution of flagella and in the appearance of new *Salmonella* serovars [18]. The evolution of P pili in uropathogenic *E. coli* has been examined by the same methodology. These studies showed that horizontal gene transfer also plays a role in the evolution of these virulence factors [19].

MEE has also been applied to study the epidemiology of resistance to antimicrobial agents. The particular case of *Streptococcus pneumoniae* will, as an example, be discussed later in this review. In the field of mycology, we have recently shown by use of MEE [17] that the appearance of resistance against the widely used antifungal agent fluconazole in HIV-infected patients was not related to the spread of one or a few resistant clones among patients. In the population studied, the appearance of resistance to fluconazole was related to a decrease in susceptibility of the infecting or colonising *C. albicans* clone without strain replacement. In addition, and thanks to MEE, we could show that the appearance of resistance to fluconazole was not restricted to a limited group of genetically closely related strains, but was observed in a large variety of genotypes, thus suggesting that under certain circumstances, any *C. albicans* strain could potentially become resistant to fluconazole. These results were in agreement with studies performed using different approaches and may have important consequences for the treatment of patients. Therapeutic protocols should be developed to avoid the emergence of numerous resistant *C. albicans* strains which may be later transmitted from one patient to another. Therapy could, for example, aim at eradication of an infecting strain by use of prolonged high doses treatment rather than trying to contain the infectious agent which may become resistant when submitted to repeated low dosage therapy.

On the other end of the spectrum of population structures, panmictic species (showing random recombination of genetic material) include not only diploid organisms with a sexual reproduction cycle, but also bacteria like *Neisseria gonorrhoeae* [14,20]. A behaviour similar to that of a sexual organism is striking in a bacterium, but is probably the consequence of the commonly accepted natural competency of this species for transformation. When a panmictic population structure is encountered, epidemiological tracing and estimation of genetic relatedness between isolates become highly questionable [12]. Under such circumstances, one has to question the significance of epidemiological typing and tracing. Genetic relatedness between isolates described by some authors for *N. gonorrhoeae* is just a relatedness between separate individuals without phylogenetic significance or any implications for associations between the genotypes evidenced by MEE and other characteristics of medical relevance like serotype and resistance to microbial agents. In such a case, each population should be considered as a whole and not as a collection of separate clones. This fact highlights the importance of a population genetics approach as offered by MEE for the study of a pathogenic microorganism before attempting any blind application of molecular typing methods for epidemiological purposes.
An intermediary kind of population structure between clonal and panmictic has been recently proposed by Maynard Smith and collaborators [14]: the epidemic population. The analysis of epidemic populations by MEE shows that they are basically nonclonal, but that selectively advantaged strains (particularly virulent, adapted to adverse environmental conditions, or resistant to antimicrobial agents) may become geographically widespread and have a relatively long persistence in a population before genetic exchange with other members of their species disrupt their apparent clonal spread. This situation seems, for example, to occur in the species Neisseria meningitidis. This species had been previously considered as clonal [21], with a strong tendency to cause epidemics or to present endemic situations in which a major clone or group of closely related types is predominant [22]. A recent analysis of a large set of MEE data by Maynard Smith [14] with a newly developed analytic technique clearly suggests that N. meningitidis presents in fact, a nonclonal population structure with a strong tendency to epidemic spread, thus mimicking at first look, a clonal situation. Combined studies using MEE, serotyping and molecular analysis of penicillin binding proteins were very successful in elucidating the possible mechanisms leading to the emergence of penicillin resistant and multiresistant S. pneumoniae. Muñoz and collaborators [23] showed that a multiresistant S. pneumoniae ET frequently encountered in Spain spread at the end of the eighties to the United States, and later work by other authors confirmed the further expansion of this ET through the USA [24]. An even more explosive spread of a single multiresistant ET was demonstrated during the same period in Iceland [25]. In a subsequent work using the same techniques, Muñoz and coworkers found evidence that penicillin resistance in S. pneumoniae has emerged independently multiple times and that the corresponding resistant ETs can spread locally or globally over several continents [26]. This propensity to epidemic spread has long hidden the real population structure of S. pneumoniae. A recent MEE study [27] using the approach proposed by Maynard Smith and collaborators [14] has shown that this microorganism presents in fact an epidemic population structure. This finding is again in agreement with the commonly accepted natural competency of this species and gives a good explanation for the lack of correlation between genotypes obtained by MEE and serotypes or results obtained by other typing methods [27]. These results once more stress the importance of fundamental population genetic approaches for the study of epidemiology and ecology of microbial pathogens.

4. MEE in microbial systematics

In clonal species, MEE data can be used to assess the degree of genomic relatedness among isolates and lineages. Based on the allelic make up of each electrophoretic type, matrices of genomic relatedness (or respectively genetic distances) can be calculated [5]. These matrices can be used to generate graphic representations of the relatedness between isolates or groups of isolates, either in the form of trees and dendrograms or in 2 or 3 dimensions by principal component analysis. Using these methods, MEE has been shown to correlate well with other techniques generally applied in systematics [28]. Thus, MEE is a good alternative to the cumbersome DNA/DNA hybridization experiments for preliminary comparison of groups of strains or for the analysis of large numbers of isolates. MEE data generally allow an accurate assignment of isolates into distinct genomic groups like species and subspecies. However, the lack of sufficient similarity among species usually does not allow us to assess the relatedness between species, even within the same genus.

For instance, MEE has been used extensively in the case of the genus Listeria to corroborate the data previously generated by DNA/DNA experiments [29]. Listeria species are phenotypically very similar and only a few criteria are available for distinguishing one from another. Therefore, MEE brought a welcome confirmation to the classification obtained by DNA reassociation experiments. Our results using MEE, ribotyping and DNA homology estimations also allowed us to definitively demonstrate that Listeria grayi and Listeria murrayi in fact represent two biotypes of the same unique species [30]. Finally, MEE allowed us to delineate two genetically clearly distinct subspecies within the species Listeria ivanovii [31]. MEE has also been useful in species delineation within several other bacterial genera with
only a few diagnostic phenotypic criteria or a lack of concordance between phenotypic and genotypic classifications. This was the case for the particularly complex genus *Aeromonas*, in which the genetic and phenotypic species classifications do not match. MEE analysis has been shown to be in broad agreement with the genospecies clustering obtained by DNA/DNA hybridisation experiments [32]. Based on their MEE results, Altwegg and collaborators could even propose a scheme for genospecies identification by electrophoretic analysis of a very limited set of metabolic enzymes to complement insufficient phenotypic criteria [32].

MEE studies have led to the delineation of several cryptic species within phenospecies thought to be homogenous. Such cryptic genospecies could often later be associated with characteristics of medical relevance and phenotypic criteria were subsequently found for their identification. The discovery of three genospecies within *Legionella pneumophila* by Selander and collaborators [33] was the first illustration of the power of MEE in such cases. Later, several other species of medically important microorganisms could be delineated using the same approach. Three genomic groups have for instance been evidenced by MEE within the species *Borrelia burgdorferi sensu lato* with different geographic distribution [34]. The existence of these three groups has since been confirmed by DNA/DNA reassociation experiments [35] and they are now recognised as the three distinct species *B. burgdorferi sensu stricto*, *Borrelia garinii*, and *Borrelia afzelii*. Further studies have confirmed the suspected association of these genospecies with particular clinical syndromes [36]. The existence of several additional genomic groups within the genus *Borrelia* has been newly confirmed or suggested by MEE [37].

In the field of mycology, we have recently identified by MEE a genomic group genetically distinct from *Candida albicans* but phenotypically very similar [38]. This genomic group is probably identical to the newly described species *Candida dubliensis* [39]. The strong association between this genomic group and HIV-infected drug addicts clearly shows its epidemiologic significance. In the field of parasitology, MEE has for instance allowed researchers to confirm the division of the species *Entamoeba histolytica* into pathogenic and non-pathogenic genomic groups [40]. This division had been suspected previously, but only the MEE analysis using a sufficient number of enzymes has been able to ascertain its validity in terms of systematics.

### 5. Clones with particular characteristics

In clonal populations, MEE allows the identification of ETs or restricted groups of genetically closely related types within a species, which can be associated with particular characteristics. These clones (in a broad sense) can subsequently be studied in more detail for the factors responsible for their particular nature and for their history and epidemiology. Many cases are known of such epidemiologically and medically important clones, which were first recognised with the help of MEE [22].

The *E. coli* serotype O157:H7 is well known for its frequent involvement in epidemics leading to enterohemorrhagic colitis and hemolytic uremic syndrome. MEE studies by Whittam and collaborators [41,42] have shown that strains of serogroup O157 are genetically diverse and suggested that the serovar O157:H7 forms a widely spread clone that did not recently derive from another O157 serotype, but possibly shared a common ancestor with serovar O55:H7 and evolved to a highly pathogenic clone by acquisition of new virulence factors by horizontal transfer [43].

In *L. monocytogenes*, MEE was used to identify an ET clearly more frequently associated with epidemics than others (ET1 in reference [44]). We have also shown that this same ET is very frequently associated with sporadic listeriosis cases in humans (Fig. 2) and animals [45]. ET1 has therefore been suspected to have a higher degree of pathogenicity than other *L. monocytogenes*. However, no distinctive pathogenicity-associated feature or particular characteristic of any known virulence factor has been identified to date, which could explain the frequent involvement of the clone marked by the ET1 in clinical listeriosis. Certain other clones of *L. monocytogenes* rarely found in clinical cases are very frequently found in meat products [45] or in fish.
products [46]. Such ETs may present a particular adaptation to meat and fish products or to their production environment. These data show that very restricted clones of *L. monocytogenes* marked by a single ET may present characteristics making them ecologically or epidemiologically clearly different from other clones of this species. Similar phenomena have been demonstrated by MEE in several other bacterial species.

A last striking example of particular clones is represented by *Staphylococcus aureus*. Musser and collaborators [47] have shown by MEE that many genetically unrelated *S. aureus* clones are able to produce the toxic shock syndrome toxin. However, a single clone was responsible for the vast majority of toxic shock syndrome cases of urogenital origin. This same ET was found as a major clone in the urogenital tract of healthy female carriers. The authors concluded that the clone marked by this ET is probably particularly well adapted for the colonisation of the urogenital tract and the human vagina, thus giving a possible explanation for its frequent involvement in menstrual toxic shock syndrome.

### 6. Epidemiological subtyping with MEE

Since MEE usually allows the identification of numerous types within a microbial species, it can be used for epidemiological tracing. The use of MEE for simple epidemiological typing at a small scale or local level without exploitation of the resultant population genetics data is somewhat disproportionate. Other more recent or simpler molecular typing methods [48] may be equivalent to or often more efficient than MEE in discriminating between epidemiologically unrelated isolates [49]. Simple methods like plasmid fingerprinting, microrestriction enzyme analysis, ribotyping and other RFLP-based techniques may be better suited for this purpose and for applications not requiring an estimation of the genetic relationships among isolates. However, MEE may represent a useful tool for the confirmation of results obtained with these methods in particular situations. Due to chromosomal rearrangement, subtypes obtained by RFLP-based methods may sometimes be relatively unstable. This leads to a relative uncertainty when trying to discriminate between genetically closely related isolates. In such cases, the slightly less discriminatory, but more stable ETs distinguished by MEE may be of great help. This was, for example, the case in a recent study that we performed on the transmission of *C. albicans* between HIV-infected sexual partners [50]. The frequent chromosomal rearrangements observed in this yeast often affect the RFLP patterns obtained with the repetitive Ca3 probe [51]. The concomitant use of MEE and Ca3 fingerprinting allowed us to show that isolates from sexual partners were more likely to belong to the same clone than isolates from unrelated patients. We could therefore conclude that transmission of *C. albicans* between HIV-infected partners probably occurs frequently. This conclusion would not have been possible by the use of RFLP based methods alone. In addition, MEE also represents an invaluable tool in large scale studies, particularly those for which other epidemiological markers may be unsatisfactory or evolving too rapidly. Beside helping to differentiate epidemiologically unrelated isolates, MEE allows the identification of clusters of genetically related strains (also called families or complexes by some authors) and to study the development of longterm or large epidemics. For instance, Caugant and coworkers could evidence the spread of a cluster of epidemic-associated serogroup B *N. meningitidis* strains over several continents during the seventies and eighties [52]. In an extensive study using serogroup A *N. meningitidis* strains from different countries Olyhoek and collaborators could also document several epidemic waves and at least two distinct pandemics caused by *N. meningitidis* during the present century by use of MEE [53]. Numerous subsequent investigations on *N. meningitidis* and on other microorganisms have clearly demonstrated the yet unsurpassed usefulness of MEE for large scale epidemiological studies.

### 7. Future of MEE in medical microbiology

Several typing methods may advantageously replace MEE in the future for different applications.
Macrorestriction analysis of genomic DNA by pulsed-field gel electrophoresis (PFGE) has recently emerged as a very popular typing technique [54]. Since a significant part of the differences between isolates observed with PFGE is apparently due to genetic rearrangements rather than to point mutations [55], the use of this technique should however be restricted to epidemiological tracing and not extended to assessment of genetic relationships. No studies have been performed to date to extensively compare the discriminatory power of MEE with that of PFGE, but many reports show that PFGE is highly discriminatory. We could, for example, distinguish 29 subtypes within a single L. monocytogenes ET defined by MEE [56]. PFGE will therefore probably advantageously replace MEE in the future for simple epidemiological tracing of pathogenic microorganisms. Random amplification of polymorphic DNA (RAPD; [57]) or arbitrarily primed PCR (AP-PCR; [58]) has been widely used in recent years for epidemiological typing of many microorganisms and is a more versatile tool than macrorestriction analysis. This method is comparable or even superior to MEE in terms of ability to discriminate epidemiologically unrelated isolates [49,59–61]. In addition, RAPD can also be used for population genetic studies similar to MEE, and in clonal species RAPD and MEE have been shown to give well correlated results [59,60]. However, many concerns have been expressed about the reproducibility of RAPD results [62–64]. A sufficient degree of intralaboratory reproducibility should be obtainable by a careful experimental design, but interlaboratory reproducibility may remain problematic for this technique and could render the comparison of results from different origins difficult if not impossible. To our knowledge, no data have yet been published on the interlaboratory reproducibility of MEE using unified protocols, but there is no doubt that standardised electrophoresis conditions and interpretation schedules using a set of reference strains should provide a satisfactory level of inter- as well as intra-laboratory reproducibility for MEE not actually possible with RAPD. Finally, the newly developed AFLP method [65] could also represent an interesting alternative to MEE. Recent publications show a great potential for this technique in epidemiological tracing [66] and in systematics [67,68].

Several other DNA-based methods may in the future also challenge MEE in the field of population genetics. Indirect sequence analysis of amplified DNA fragments by single strand conformation polymorphism [69] could, for example, represent an interesting alternative to MEE, but at the DNA level. The recent developments in the DNA-sequencing techniques and in their automation may also make direct sequencing of sets of well chosen DNA fragments for numerous isolates possible at affordable costs and within acceptable time frames. This would ultimately lead to an even more profound understanding of population structures and of evolution of microbial pathogens than MEE. These techniques are however far from large scale applicability today. Meanwhile, MEE with its wide field of applicability ranging from the genospecies level to the level of restricted clones will remain the gold standard to which every newly developed technique will have to be compared.

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