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Multilocus Sequence Typing

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Abstract

Multilocus sequence typing (MLST) was first proposed in 1998 as a typing approach that enables the unambiguous characterization of bacterial isolates in a standardized, reproducible, and portable manner using the human pathogen *Neisseria meningitidis* as the exemplar organism. Since then, the approach has been applied to a large and growing number of organisms by public health laboratories and research institutions. MLST data, shared by investigators over the world via the Internet, have been successfully exploited in applications ranging from molecular epidemiological investigations to population biology and evolutionary analyses. This chapter describes the practical steps in the development and application of an MLST scheme and some of the common tools and techniques used to obtain the maximum benefit from the data. Considerations pertinent to the implementation of high-capacity MLST projects (i.e., those involving thousands of isolates) are discussed.

Keywords

High-throughput sequencing; MLST; population genetics; sequence types

1. Introduction

Multilocus sequence typing (MLST) (1) combined a number of technical and conceptual developments of the last two decades of the 20th century to provide a universal, portable, and precise means of typing bacteria (1-3) . The approach owed much to the pioneering technique of multilocus enzyme electrophoresis (MLEE), from which it acquired its name (4). A key conceptual development was the recognition that bacteria do not necessarily have a clonal population structure (5, 6), leading to the realization that patterns of genetic exchange among bacteria, and therefore of descent, could only be resolved by the analysis of nucleotide sequence data from multiple locations of the chromosome (7) . Developments in high-throughput nucleotide sequence determination and analysis permitted the generation of definitive genetic data from any locus on the chromosome of multiple isolates (8) . An advantage of nucleotide sequence data is that they can be disseminated via the Internet, particularly the World Wide Web (9, 10) .

The first MLST scheme developed was for the human pathogen *Neisseria meningitidis* (1), largely as a result of the leading role that studies of this organism had played in the development of the more sophisticated appreciation of bacterial population structure

(11-14). It is noteworthy that the success of this scheme was, to a great extent, due to its immediate acceptance by the wide community of researchers working on pathogenic *Neisseria*. This was due to the fact that the scheme was developed and promoted by a consortium of leading researchers in the fields of meningococcal epidemiology and population biology. Cooperation and collaboration continue to be cornerstones of successful MLST schemes.

MLST has since been applied to a number of different bacteria and eukaryotic organisms as a tool for the epidemiological analysis and surveillance of pathogens as well as to investigate their population structure and evolution. MLST has also been deployed in studies of the population structure of nonpathogenic bacteria (2).

MLST provides a number of advantages over other typing approaches. First, it uses sequence data and can therefore detect changes at the DNA level that are not apparent by phenotypic approaches, such as serotyping, and by MLEE that uses the migration rate of proteins in starch gels. Second, it is a generic technique that can be readily reproduced and does not require access to specialized reagents or training. Third, modern methods of direct nucleotide sequencing, based on the polymerase chain reaction (PCR), do not require direct access to live bacterial isolates or high-quality genomic DNA. These techniques can be performed on killed cell suspensions, avoiding all the difficulties associated with the transport and manipulation of pathogens, or on clinical samples, such as the cerebrospinal fluid or blood of a patient undergoing antibiotic therapy, from which a live bacterial isolate might be difficult to obtain. Fourth, the data generated are fully portable among laboratories and can be shared through-out the world via the Internet. Finally, the Internet can also be used to disseminate MLST methods, providing standardization of approaches (2).

This chapter describes the principles behind the development and application of an MLST scheme using the methods deployed in the *Neisseria* scheme as an example. In particular, the upscaling of MLST to enable the cost-effective typing of many hundreds or thousands of isolates is discussed. The general principles are applicable to essentially all bacteria, although the utility depends on the diversity of the population under investigation and the question asked. The chapter concludes with an overview of some of the approaches available for the basic analysis of MLST data.

2. Materials

2.1. Isolate Collection

A representative sample of the population for which the scheme is to be developed (*see* Note 1).

2.2. Preparation of Killed Cell Suspensions

1. Freshly grown plates of bacterial cultures.

¹The isolates examined must be carefully chosen with a number of criteria in mind: They should represent the known genetic diversity of the population analyzed (which itself should be carefully defined); they should represent a variety of sources or environments from which the organism is often isolated; and they should be collected from a variety of geographic locations and an appropriate time frame.

2. Boiling water bath.
3. Sterile phosphate-buffered saline (PBS).
4. 1.5- to 2.0-mL screw-capped microcentrifuge (Eppendorf) tubes (not double-walled or skirted tubes).
5. Sterile swabs/loops.

2.3. PCR Amplification of Gene Fragments

1. DNA template.
2. Forward and reverse primers.
3. DNA polymerase enzyme (*Taq* polymerase).
4. Deoxyribonucleoside 5'-triphosphates (dNTPs).
5. Buffer solution (supplied with the enzyme).
6. Magnesium chloride (supplied with the enzyme).
7. Microtiter plates resistant to high temperatures or 0.6-mL capacity microfuge tubes.
8. Thermocycler.

2.4. Gel electrophoresis

1. Agarose.
2. Ethidium bromide: 10 mg/mL stock solution.
3. Loading buffer.
4. TBE buffer: A 10× stock (0.89 M Tris-HCl, 0.89 M boric acid, 20 mM ethylenediaminetetraacetic acid [EDTA], pH 8.3).
5. Power source.
6. UV transilluminator.

2.5. PCR Product Purification

1. 1.5-mL microcentrifuge tubes.
2. Polyethylene glycol (PEG) 8000.
3. Sodium chloride.
4. Ethanol 70%.
5. Benchtop centrifuge.

2.6. Sequencing Reactions

1. Purified PCR products (DNA template).
2. Forward and reverse primers.

3. Sequencing kit containing DNA polymerase and labeled dNTPs.
4. Microtiter plates or 0.6- μ L tubes.
5. Thermocycler.
6. DNA sequencer.

2.7. Purification of Sequencing Products

1. 1.5-mL microcentrifuge tubes.
2. 3 M sodium acetate, pH 4.6.
3. Ethanol, 95% and 70%.
4. Benchtop centrifuge.

3. Methods

3.1. Killed Cell Suspensions

1. Heat the water bath until it boils.
2. Clearly label the screw-capped microcentrifuge tubes. Ensure that these labels will not come off during the heating step.
3. Dispense 0.5 mL of PBS in each microcentrifuge tube.
4. Make very thick suspensions of organisms by sweeping colonies from each culture plate using a swab or a loop and emulsifying in the PBS in the tubes.
5. Place the tubes in the boiling water bath and leave for 20 min.
6. Store the samples at -20°C . These samples are, in principle, killed and stable at room temperature. Once lack of viability has been confirmed, they can be handled in the laboratory and distributed as noninfectious material (*see* Note 2).

3.2. PCR Amplification (see Note 3)

1. Initialization: The reaction mix is heated to 94°C for 1 min to denature the DNA.
2. The following steps are repeated for 25–30 cycles:
 - a. Denaturation at 94°C for 30 s.
 - b. Primer annealing at $50\text{--}60^{\circ}\text{C}$ for 30 s. This allows the primers to bind to the template DNA.
 - c. Extension at 72°C . During this step, the *Taq* polymerase uses the dNTPs to synthesize a new DNA strand complementary to the template. The

²The crucial step in this method is the rapid inactivation of cellular nucleases once the cells have been lysed.

³In an MLST scheme, PCR conditions are ideally the same for all loci. This should be straightforward if primers are designed to have similar melting temperatures T_m and if the DNA fragments to be amplified are of similar lengths. Optimization is likely to be needed for novel primers.

duration of this step depends on the length of the fragment that is to be amplified.

3. Final elongation at 72°C for 5–10 min to ensure that all the fragments are fully extended.
4. The reaction should be held at 4°C until removed from the thermocycler.

3.3. Agarose Gel Electrophoresis (see Note 4 and ref. 15)

1. Prepare a 1% (w/v) agarose gel by adding 1 g of agarose to 100 mL of TBE buffer.
2. Heat in a microwave until boiling.
3. Leave it to cool for 2–3 min.
4. Add 5 µL of ethidium bromide.
5. Insert the gel comb and wait until is solid.
6. Fill in the electrophoresis tank with TBE.
7. Insert the gel into the tank and remove the combs.
8. Mix 5 µL of PCR product with 2 µL of loading buffer.
9. Connect the gel tank to the power source.
10. Set the voltage to 140 V and leave it running for 15–20 min.
11. Visualize the gel using a UV transilluminator.

3.4. Purification of Amplicons (see Note 5)

1. Transfer the contents of each PCR tube into labeled 1.5-mL Eppendorf tubes. If microtiter plates are used, this step can be omitted.
2. Add 60 µL of 20% (w/v) PEG 8000, 2.5 M sodium chloride to each tube and mix. Incubate for 30 min at room temperature.
3. Pellet the PCR products by spinning in a centrifuge at maximum speed for 15 min. For microtiter plates, spin for 1 h at 2,750 *g* .
4. Discard the supernatant and wash the DNA pellet by adding 0.5 mL of 70% ethanol and spin at maximum speed for a further 5 min. For microtiter plates, add 150 µL of 70% ethanol and spin for 10 min at 2,750 *g* . Repeat this step twice when using plates.
5. Discard the supernatant and dry pellets in the vacuum dryer. Microtiter plates can be dried by spinning upside down for 1 min at 500 *g* .

⁴Standard agarose gel electrophoresis can be employed to check that the amplification reactions have been successful and that amplicons of the expected size have been produced. It is recommended to check all the samples during the optimization period, but when the MLST scheme is fully developed and routinely applied on a large scale, only occasional verification is necessary (15).

⁵A variety of methods for purification are available, including many commercial kits. However, the purification method described here is an effective and inexpensive noncommercial method based on sodium chloride and PEG differential purification.

3.5. Nucleotide Sequence Extension Reactions (see Note 6)

1. Mix the primer, template, and sequencing reagents in the optimized proportions.
2. Perform the extension reactions in a thermocycler, first conducting denaturation at 96°C for 1 min.
3. The following steps are repeated for 25 cycles: 96°C for 10 s, 50°C for 5 s, 60°C for 40 min.
4. Maintain the reaction at 4°C until removed from the thermocycler.

3.6. Purification of Sequencing Products

1. Transfer the contents of each PCR tube into labeled 1.5-mL Eppendorf tubes. If microtiter plates are used, this step can be omitted.
2. Add 2 μ L of 3 M sodium acetate and 50 μ L of 95% ethanol to each tube and mix. Incubate for 45 min at room temperature.
3. Pellet the PCR products by spinning in a centrifuge at maximum speed for 15 min. For microtiter plates, spin for 1 h at 2,750 g .
4. Discard the supernatant and wash the DNA pellet by adding 0.5 mL of 70% ethanol and spin at maximum speed for a further 5 min. For microtiter plates, add 150 μ L of 70% ethanol and spin for 10 min at 2,750 g .
5. Discard the supernatant and dry pellets in the vacuum dryer. Microtiter plates can be dried by spinning upside down for 1 min at 500 g .
6. For separation and detection of extension products, *see* Note 7.

3.7. Data Management

3.7.1. Data Assembly—A variety of commercial and open source software packages are available for the assembly and editing of sequence chromatograms into compiled edited sequences, including the well-known Staden and GCG packages (16, 17) . Specialist software for the compilation and analysis of MLST data is also available, for example, the START software package (18) . These packages allow many hundreds or even thousands of samples to be processed cost-effectively and rapidly. Inexpensive Linux-based software (19), as well as commercial solutions, are available. The use of Internet-based databases and analytical tools designed for MLST analysis can automatically designate sequence type (ST) and clonal complex as well as facilitate storage and access to the data via Internet.

⁶It is an absolute requirement for accurate sequence determination that sequence information from both DNA strands is used to compile the final sequence, so PCR reactions “forward” and “reverse” are required for each DNA molecule to be sequenced. The reactions are easily performed with proprietary kits that contain all of the necessary components, requiring only template DNA and specific primer to be added. Some local optimization is likely to be required for the primers and reagents used.

⁷A variety of commercial instruments is available for the separation of extension reaction products, and a description of their operation is beyond the scope of this chapter. In most cases they are capillary based and generally operated by central sequencing facilities as they are high-cost assets that, to be cost-effective, have to be used on very large numbers of samples, usually representing a wide variety of applications. Although smaller-scale instruments suitable for the use by single laboratories are available, they are usually much more expensive to run. Commercial companies also offer sequencing services.

The sequence type analysis and retrieval system (STARS) is specifically designed for the assembly of MLST data (<http://www.cbrg.ox.ac.uk/~mchan/stars/>). It uses PREGAP4 and GAP4 from the Staden package (16) to automatically assemble a large number of sequences, which can be retrieved and edited. For known alleles and STs, designation can be done directly from the STARS interface by interrogating an MLST database.

3.7.2. Data Storage—The maintenance of curated, Web-accessible databases is a key feature of MLST schemes. These databases act as dictionaries that allow bacterial isolates to be compared worldwide (2). Database management is therefore central to the endeavor. The key part of MLST databases is comprised of the allele sequences linked to MLST allele numbers for each locus and the definition of STs. In some cases, it may be appropriate to include information on higher-order organization of STs into clonal complexes or lineages in this database as is done with the *Neisseria* MLST Allelic Profile/ST Database. These data can then be linked to isolate databases that contain isolate specific information. It is important that there is a separation between the databases containing the allele and ST data and isolate data as many isolates will contain the same alleles or STs (9).

3.8. Data Analysis

3.8.1. Analysis of MLST Data—The first question to be addressed with an MLST data set is whether the data conform to the clonal model of population structure. Clonal population structure is an inevitable consequence of asexual reproduction combined with diversity reduction events, such as periodic selection and sequential bottlenecks (20). If an organism is clonal, then the analysis is greatly simplified as conventional phylogenetic trees can be employed. Clonality can be investigated by the congruence test (21), which is based on the observation that, in a clonal population, the phylogenetic signal observed at different loci is the same or congruent (22).

Most bacteria that have been analyzed by MLST are, however, nonclonal by the congruence test. For such organisms the clonal complex is a useful concept that groups genetically related organisms. Clonal complexes comprise groups of related STs that are likely to derive from a common ancestor. Currently, the designation of clonal complex is pragmatic and to an extent varies with different bacteria, but the important issue is that the grouping is consistent with what is known and understood about the genealogy of the organism. The BURST (based upon related sequences) algorithm is a rapid and effective algorithm that can be used to assign the central genotype of clonal complexes. The eBURST program (23) groups STs into groups according to user-defined criteria of a number of alleles in common to at least one other member of the group. The central genotype of a BURST group will be the one with the highest number of singlelocus variants (SLVs). This will often coincide with the one most frequently isolated and therefore gives some biological meaning to the future designation of the clonal complex. The eBURST program and instructions can be found at <http://eburst.mlst.net/>. A number of clustering algorithms, such as the unweighted pair group method with arithmetic mean (UPGMA) (24) or split decomposition (25) can be used to cluster STs and reinforce the results obtained using eBURST.

3.8.2. Applying the Clone Complex Model—It is possible to rationalize the clonal complex structure of many bacteria in terms of the “epidemic clone model” (5) of bacterial population structure or modifications of it. Within this frame-work, high prevalence of a single ST indicates the presence of a fast-spreading new clone from which variants are developing. In the absence of a formal means of defining such clones, it is necessary to implement a rational definition that will command support from the scientific community analyzing these bacteria. It is advisable to designate a committee of experts who ultimately decide on the management and nomenclature issues raised by the scheme.

3.9. High-Throughput MLST

One of the great advantages of MLST is its scalability from a single bacterial isolate to many hundreds or even thousands of samples. Upscaling of MLST is essential for large-scale studies and brings with it appreciable advantages in terms of reducing costs. Automation reduces staff input, and bulk purchase of reagents brings substantial cost savings. While automation brings substantial benefits, it does require substantial commitment and investment. During the setup process the various sections of the data production and analysis pipeline have to be analyzed and kept under review; potential bottlenecks can then be identified and handled. Any process is only as efficient and rapid as its least-efficient and slowest step. PCR and sequencing reactions can be automated by investing in a robotic platform that saves personnel time and minimizes error (26, 27) or at least ensures that any error is deterministic rather than stochastic. A number of fast and reliable methods exist for the purification of amplification products that can be incorporated into the robotic platform, although consumables for these types of systems are often expensive. The PEG precipitation for PCR products and sodium acetate/ethanol precipitation for sequence reactions are highly cost-effective, but are time consuming and require investment in centrifugation equipment capable of sedimenting material in microtiter plates.

Optimization of the sequence reactions and the use of a centralized sequencing facility can further reduce costs as the use of reagents can be minimized, and costs can be further reduced by bulk purchase (28). If automation is to be used, it is important to recognize that the processes are more akin to those found in industrial rather than conventional biological research organizations. Robotic equipment works most effectively when it is regularly used to perform highly repetitive operations. Once the equipment is working on a given application, a process that often requires appreciable investment of time and effort, temptation to further improve operation by minor modification should be resisted. Such attempts prevent the exploitation of the equipment efficiently and are at least as likely to degrade as to enhance the performance of the equipment.

3.10. Applications of MLST Data

3.10.1. Application to Public Health—Public health laboratories use MLST routinely for the characterization of clinical specimens (29,30). For the meningococcus, for example, the information obtained has proven to be invaluable for the understanding and management of disease outbreaks (31,32), epidemiological surveillance (33,34), and the monitoring of public health interventions. Its application to clinical specimens has obvious implications for

diagnosis and clinical management of cases caused by an organism that is notoriously difficult to isolate microbiologically from patients undergoing antibiotic therapy (35-37).

3.10.2. Evolutionary and Population Genetic Analyses—MLST data have been used in a wide variety of applications, including evolutionary and population analysis of bacterial species, but to date they have been mostly used in molecular epidemiological studies of bacterial pathogens. Molecular epidemiology employs genetic techniques to characterize isolates of infectious agents or identify their presence and characteristics from clinical specimens. By this means their distribution and spread can be monitored, and if necessary, health interventions can be implemented. MLST has been applied to many bacteria, as recently reviewed (2). MLST data can also be used to investigate the population structure of bacterial populations at different levels (e.g., temporal stratification or geographic distribution) as this can help to understand the transmission route of the infectious agent (38). For this purpose, the analysis of molecular variance (AMOVA) (39) can be used to calculate the F statistic (F_{ST}) (40), which measures the amount of genetic exchange that takes place among different groups of organisms. The Mantel test can be used to investigate the correlation between genetic and geographic distance, that is, whether isolates obtained from geographically close locations are more closely related to those found on more distant geographic areas (38). Both tests can be easily implemented using the Arlequin software package (39), which can be downloaded from <http://lgb.unige.ch/arlequin/>

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