INTRODUCTION

*Neisseria gonorrhoeae*, an exclusively human pathogen, causes 87.5 million new gonorrhea infections worldwide annually (195). Although gonococcal infections are treated with antibiotics, the microorganism is now resistant to most antibiotics and no vaccines are available. Gonorrhea can cause severe complications, such as epididymitis in men and pelvic inflammatory disease in women. Gonococcal infection can also lead to complications in newborns, such as eye infections (gonococcal ophthalmia neonatorum) during passage of the newborn through the birth canal of an infected mother, which can result in blindness (13, 66, 107, 150). Importantly, gonorrhea is associated with the transmission of other sexually transmitted infections (STIs) and with human immunodeficiency virus (HIV) infections (53a).

Identification of cases and effective treatment with antibiotics are the mainstay approaches for the prevention and control of gonorrhea. However, there are major concerns worldwide regarding the renewed rising incidence of gonorrhea in many countries, coupled with the high prevalence of resistance to antimicrobial agents previously recommended for treatment (i.e., penicillin, erythromycin, tetracycline, and ciprofloxacin) (32, 72, 158, 160) and coupled with reduced susceptibility and/or resistance to presently recommended antimicrobial agents such as azithromycin and extended-spectrum cephalosporins (i.e., cefixime and ceftriaxone). Therefore, gonorrhea may become untreatable in certain circumstances, and the organism has emerged as a “superbug” (5, 160). International efforts to gather information on emerging trends in antimicrobial susceptibility coupled with regional, national, and international surveillance of the epidemiological characteristics and spread of *N. gonorrhoeae* have become a public health priority. However, the possibility of performing reliable phenotypic an-
Timicrobial susceptibility testing has been decreased because nucleic acid amplification tests (NAATs) are rapidly replacing culture for the diagnosis of gonorrhea (51, 159, 160, 183). Therefore, comprehensive knowledge regarding the genetic and molecular bases for antimicrobial resistance and subsequent development of genetic methods for determination of antimicrobial resistance in *N. gonorrhoeae* are critical.

There has been a long history of typing isolates of *N. gonorrhoeae* both for epidemiological purposes and for investigating the transmission of antibiotic-resistant isolates. As purposes for typing isolates differ, it is crucial to choose the most effective typing method(s) for answering specific questions. Ideally, a typing method should display sufficient discrimination to differentiate between isolates from unlinked sources and be sufficiently stable to identify linked cases from the same source; i.e., each strain will appear different unless part of a transmission chain. Typing methods can be used for a variety of purposes: to understand phylogeny (evolution) and bacterial population genetics, to identify specific strains spreading globally in specific populations and/or in core groups, to identify temporal and geographic changes in strain types as well as the emergence and transmission of individual strains, to establish strain identity/difference in contact tracing or test of cure, to confirm/disprove treatment failures, to resolve medico-legal issues such as sexual abuse, and to confirm presumed epidemiological connections or discriminate isolates of suspected clusters and outbreaks. Strain typing coupled with antimicrobial susceptibility data helps in a better understanding of the transmission of specific antibiotic-resistant strains. Ultimately, such information can be applied to design different public health preventive measures and interventions.

A variety of typing methods have been used to differentiate *N. gonorrhoeae* isolates. Antimicrobial susceptibility patterns have been used to “type” strains based on susceptibility profiles, although the ability of this method to identify transmission chains is low. Some earlier phenotype-based typing methods, such as auxotyping and/or serovar determination, may still be valuable as primary epidemiological markers. Serovar determination has a relatively high discriminatory ability. However, the use of auxotyping and serovar typing is limited due to the unavailability of reagents, the high technical expertise required, and the relatively high cost. Genotyping methods are now the methods of choice for typing gonococcal isolates because they are more discriminatory, reproducible, objective, and reliable despite their various performance characteristics (see below).

It is not possible to utilize a single typing method to accurately and precisely reveal answers to all questions pertaining to contact tracing, characterization of clones, investigation of smaller clusters of infection, identification of strains from large core groups, characterization of community epidemics, phylogeny, and bacterial population genetics. The questions asked in relation to specific clinical, epidemiological, or scientific situations should guide the selection of the most effective typing method. Furthermore, typing may involve resolving issues of short-term epidemiology (microepidemiology; days to a maximum of a few years), long-term epidemiology (many years to decades), and/or global epidemiology (macroepidemiology).

We reviewed all available typing methods used to differentiate *N. gonorrhoeae* isolates. The aims of the present review are to describe and evaluate methods for the phenotypic and genotypic characterization of *N. gonorrhoeae* and the performance characteristics of these methods and to recommend which methods should be used in different situations. This review concludes that appropriate, validated, and quality-assured DNA sequencing methods should become the methods of choice for typing *N. gonorrhoeae* isolates worldwide, a recommendation based on all available evidence and the relatively low cost and accessibility of these methods presently.

### Typing Neisseria Gonorrhoeae Isolates

Many methods have been developed and applied to phenotypically and genotypically characterize *N. gonorrhoeae* isolates. The performance characteristics of the widely used methods to type isolates are summarized in Table 1.

Simpson’s index of diversity (41, 68, 69, 115), which numerically describes the ability of a method to discriminate between unrelated strains, is also considered in our discussions regarding the evaluation or selection of a particular typing method. In general, indices of $\geq 0.95$ (95%) are considered to be an ideal indicator of the ability of a given method to discriminate between strains. This index is influenced by sample size and the heterogeneity and clonality of the bacterial population tested. Ideally, calculations of the index should be accompanied by a critical assessment of the confidence interval. A method with a low index of discrimination should not be used to predict whether two isolates might be linked epidemiologically or to identify isolates from a core group of transmitters.

#### Traditional, Non-DNA-Based Phenotypic Typing Methods

For several decades, phenotypic methods, such as antimicrobial susceptibility profile determination, auxotyping, and serovar determination, have been used to differentiate *N. gonorrhoeae* strains (Table 1). Conclusions regarding strain type and distribution acquired using phenotypic characterization methods should be interpreted with caution due to the inherent insensitivity of these methods to delineate isolates appropriately.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing is fundamental in clinical practice for effective treatment of patients, for monitoring changing patterns of antibiotic susceptibility, for identifying emerging resistance phenotypes, and for informing the development of effective treatment guidelines. Analysis of antimicrobial susceptibility profiles (antibiograms) has low discriminatory ability and should not be used for the epidemiological characterization of strains (Table 1).

**Auxotyping.** Methods for auxotyping *N. gonorrhoeae* isolates were first described in 1973 (17, 18), and there were a number of subsequent modifications (34, 35, 63, 111, 188). Auxotypes of *N. gonorrhoeae* isolates are based on their different nutritional requirements for amino acids, purines, pyrimidines, and vitamins. This method has low discriminatory ability, is time-consuming and laborious, and requires a high level of technical expertise and interpretation. During the 1980s and 1990s, auxotype (A) and serovar (S) determination were combined to determine the A/S classes of isolates as a typing method (Table 1). The combination of these two methods provides a relatively
high level of strain discrimination. It should be noted that newer genotyping methods have a higher ability to discriminate strains and are less labor-intensive (51, 71, 80, 105, 106, 111, 112, 118, 125, 132, 166, 167, 174, 182, 196, 197).

**Serovar determination.** Serovar typing methods are based on antigenic heterogeneities of the outer membrane porin (i.e., PorB, encoded by *porB*) protein. Serovars are determined using coagglutination techniques (139) for detecting interactions between gonococcal antigens and panels of specific monoclonal antibodies (MAbs) (76). Two major schemes based on MAbs were developed: the Genetic Systems (GS) panel (80, 157) and the Pharmacia (Ph) panel (140, 141). Unfortunately, the widely used MAbs of the GS panel are no longer available. While the Ph panel of MAbs is still commercially available, its price is relatively high. The serovar typing methods have been extensively used to differentiate *N. gonorrhoeae* isolates, and they are sometimes used as controls to evaluate new genotyping methods (6, 14, 15, 24, 34, 37, 43, 49, 54, 57, 65, 66, 69, 71, 73, 80, 87, 102, 105, 106, 110, 111, 115, 118, 132, 133, 135, 141, 153–156, 162, 166–168, 170–172, 176–181, 190, 196, 197).

Serovar determination has a higher discriminatory ability than auxotyping. It is fast, easy to perform, and relatively cost-efficient. It does not require sophisticated equipment. Serovar typing also provides information on the antigenicity of expressed PorB. Although the determination of serovar may be important for a better understanding of host immune response and immune protection and, ultimately, in the development of gonococcal vaccines, the disadvantages include suboptimal discriminatory ability compared to modern genotypic methods, reproducibility issues due to the subjective interpretation of results, low specificity of some MAbs, the increasing prevalence of nonserotypeable strains, and the emergence of new serovars over time due to the ongoing evolution of *porB* (25, 57, 65, 70, 101, 117, 176, 178, 199) (Table 1). Nevertheless, serovar determination is still a valuable tool as a rapid primary marker for differentiating *N. gonorrhoeae* isolates, especially in those regions where genetic characterizations are not possible.

**DNA-Based Typing Methods**

Over the past 2 decades, a number of genetic (DNA-based) typing methods for characterizing *N. gonorrhoeae* strains have been developed which may better discriminate between strains and which have become increasingly more cost-effective and reproducible (Table 1). DNA-based typing methods include characterizing plasmids as well as determining polymorphisms in a single locus or multiple loci using a number of methods (or, potentially, the entire genome). These methods can be broadly divided into two groups: those methods involving analysis of DNA banding patterns by gel electrophoresis (gel-based DNA-based typing methods) and those based on DNA sequence analysis (DNA sequence-based typing methods). Gel-based DNA-based typing methods include plasmid content analysis or restriction fragment length polymorphism (RFLP) determination using pulsed-field gel electrophoresis (PFGE), ribotyping, and Opa typing. DNA sequence-based typing methods include full- or extended-length *porB* sequence analysis, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), and multilocus sequence typing (MLST). This review will provide evidence-based rationales so that appropriate, val-
Gel-based DNA-based typing methods. (i) Analysis of plasmid content. Analysis of plasmid content involves the characterization of either the complete plasmid content or specifically targeted plasmids in *N. gonorrhoeae* isolates. Specific plasmids have been classified based on their phenotypes, molecular weights, or DNA sequences, including cryptic and conjugative plasmids, as well as resistance determinant-containing plasmids such as the tet*M*-carrying conjugative plasmid and the family of β-lactamase-producing plasmids (16, 31, 33–42, 47, 55, 59, 91, 92, 108, 110, 120, 126, 169, 198). Plasmid content analysis has a low discriminatory ability, and occasionally there may be an issue with lack of reproducibility as plasmids may be acquired or lost from isolates. It is not recommended as a routine typing method.

(ii) RFLP analysis and PFGE. Several methods which can be characterized as a type of restriction fragment length polymorphism (RFLP) analysis have evolved. An early version of RFLP analysis was based on digestion of the entire genome by high-frequency-cutting restriction endonucleases followed by separation of fragments by polyacrylamide gel electrophoresis (PAGE) (45, 46). This method is no longer commonly used because it is laborious and not suitable for high-throughput analysis. Furthermore, the results obtained are difficult to objectively interpret and generally require manual interpretation, causing problems with reproducibility and interlaboratory comparisons.

The introduction of pulsed-field electrophoresis (PFGE) to resolve RFLP fragments on gels was an important innovation. PFGE is based on digestion of the entire bacterial genome by rare-cutting restriction endonucleases followed by separation of the resulting large DNA fragments in an agarose gel subjected to pulsed-field electrophoresis (142). This method can separate large DNA fragments (of 5 to 10 Mbp [187]) in a size-dependent manner, with relatively few bands to compare. Digestion of genomic DNA with enzymes such as SpeI and or BglII has proven to be highly discriminatory for *N. gonorrhoeae*. PFGE has been used to define the gonococcal strain populations in a particular region, to identify clusters of circulating strains, including antibiotic resistant strains (3, 24, 49, 51, 54, 71, 80, 87, 105, 111, 113, 132, 133, 135, 153, 154, 156, 170, 172, 174, 179, 182, 196, 197), and in forensic evaluations (30; M. Unemo, unpublished data). This method can distinguish subtypes within A/S classes and within genotypes determined with other highly discriminatory DNA sequence-based methods, such as full- or extended-length *porB* sequencing and NG-MAST. Therefore, PFGE is particularly a useful method to increase discrimination between isolates in specific situations, especially those involving extreme microepidemiology (49, 54, 82, 135, 153, 172, 174, 178). PFGE is reproducible, and all *N. gonorrhoeae* isolates are typeable by this method (24, 54, 71, 80, 87, 105, 111, 113, 132, 133, 135, 153, 154, 156, 170, 172, 174, 178, 192, 196, 197). The distinct disadvantages of RFLP and PFGE analysis include the requirement for a high level of technical and interpretive expertise, the potentially subjective interpretation of banding patterns on gels, the time involved for typing (several days), the lack of high-throughput analysis, and high cost. The universal applicability of PFGE-based RFLP analysis would require pronounced standardization in both national and global contexts (Table 1).

(iii) Ribotyping. Ribotyping is based on RFLP analysis of rRNA genes. Chromosomal DNA is digested with restriction enzymes, causing ribosomal genes and their adjacent regions to be widely distributed, with subsequent identification of restriction fragments by hybridization to a specific rRNA probe (74, 80, 82, 110). This method produces specific and reproducible hybridization patterns and simplifies the interpretation of results compared to that with PAGE or PFGE analysis by reducing the number of bands to interpret. However, ribotyping has little applicability for *N. gonorrhoeae* strains, as its discriminatory ability is low. Furthermore, the method is laborious, involves subjective interpretation of results, and is not practical for use outside reference laboratories. Unless expensive ribotyping equipment is purchased, the method is not suitable for high-throughput analysis (74, 80, 82, 110) (Table 1).

(iv) Opa typing. Opa typing is based on the PCR amplification of 11 *opa* genes followed by restriction endonuclease digestion with TaqI and HhaI, separation of fragments by gel electrophoresis, and subsequent visualization of the banding patterns (118). The method imparts high typeability and reproducibility and excellent discriminatory ability, even in comparison with highly discriminatory DNA sequence-based approaches (19, 20, 26, 49, 65, 71, 74, 93, 96, 104, 118, 121, 127, 174, 182, 186, 189). However, Opa typing has the same disadvantages as RFLP methods, including labor-intensiveness, subjectivity, and a need for pronounced standardization for interlaboratory comparisons. Opa typing has been used to define gonococcal populations within a geographic area, for the identification of clusters of strains (8, 9, 19, 20, 26, 49, 65, 71, 74, 93, 96, 104, 118, 121, 127, 174, 182, 186, 189), for tracing strain transmission between sexual contacts (9, 19, 74, 186), for resolving suspicions of reinfection (102), for substantiation of treatment failure (77, 102), and for detection of mixed infection (102). Opa typing has been used to increase the discriminatory power of other typing methods, including full- or extended-length *porB* sequencing or NG-MAST in specific situations, especially those involving extreme microepidemiological analysis (19, 65, 96, 104, 174, 186) (Table 1).

(v) Other PCR-based typing methods. Additional PCR-based typing methods for *N. gonorrhoeae* include amplified rRNA gene restriction analysis (ARDRA), a variant of ribotyping. For ARDRA, a ribosomal gene fragment including part of the 16S rRNA gene, the 16S-23S rRNA spacer region, and part of the 23S rRNA gene is PCR amplified, followed by digestion of PCR products with a high-frequency-cutting restriction enzyme (60, 182) and subsequent gel electrophoresis analysis. Other PCR-based analyses include (fluorescent) amplified fragment length polymorphism (AFLP) (121, 149), whole-cell repetitive element sequence-based PCR (rep-PCR) analysis (136), arbitrarily primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD) typing (14, 65, 78, 182), and multilocus variable-number tandem repeat (VNTR) analysis (MLVA) (64; R. Heymans and M. Unemo, unpublished data). These methods have not been widely used, and their disadvantages include suboptimal discriminatory ability (with the exceptions of AFLP [121] and MLVA [R. Heymans and M. Unemo, unpublished data]), labor-intensiveness, subjectivity of
interpretation, the need for standardization for international comparisons, and poor accessibility.

DNA sequence-based typing methods. (i) porB-based DNA sequence analysis. porB-based DNA sequence analysis is based on DNA sequence analysis of either an extended length of porB, which comprises most polymorphic segments, or the full length of the gene (up to nearly 1,000 bp). PorB is the antigenic target of serovar determinations, and the DNA sequencing of its encoding gene, porB, has been increasingly used, either singly or in combination with other genes, for DNA sequence-based typing of N. gonorrhoeae strains. Regrettably, the prospects for developing a genetic typing system congruent with results obtained by GS serovar determination are limited because the precise amino acid residues of PorB critical for the reactivity of many of the MAbs remain unknown (12, 23–25, 100, 176).

The full- or extended-length porB sequencing typing approaches are highly discriminatory and reproducible, all strains are typeable, and results can be compared objectively between laboratories based on DNA sequence (Table 1). In fact, a database that assigns a sequence type (ST) number based on the limited porB DNA sequence (490 bp) obtained using the NG-MAST method (see below) has been established, thereby allowing international comparisons of STs. The newly developed automated DNA sequencing technologies have reduced the cost and broadened the availability globally of sequencing technology. porB sequence typing methods have been used to describe gonococcal populations in a region and to identify clusters of strains (24, 49, 54, 58, 69, 71, 73, 79, 83, 84, 87, 95, 96, 101, 103, 104, 109, 115, 122–125, 128, 138, 153, 155, 156, 162, 165, 172–175, 180, 190–193), to trace sexual contacts (1, 9, 19, 96, 172), for investigating treatment failures (86, 119, 161, 173), and in medico-legal cases (97). NG-MAST has also been evaluated as a tool for predicting specific antimicrobial resistance phenotypes in N. gonorrhoeae isolates (124). However, this application is far from ideal. More research in determining whether certain STs are correlated with specific antibiotic resistance phenotypes and their temporal stability is needed, using a higher number of isolates that are phenotypically, genetically, geographically, and temporally diverse. NG-MAST (http://www.ng-mast.net) is most often used with cultured specimens, and the method must be optimized for potential use in all types of NAAT specimens (191).

(ii) NG-MAST. The Neisseria gonorrhoeae multiantigen sequence typing (NG-MAST) method examines the variable internal fragments of two highly polymorphic loci of N. gonorrhoeae: porB (490 bp is examined) and tpbB (390 bp is examined), which encodes subunit B of the transferrin binding protein (96). NG-MAST, which has high discriminatory power and high reproducibility and typeability, has been widely used because it is easy to perform and because a public database (http://www.ng-mast.net) can be accessed for analysis and for the assignment of discrete allele numbers and sequence types (STs). Since the cost of DNA sequencing continues to diminish, this methodology has become more accessible to many resource-challenged reference laboratories. Nevertheless, isolates having identical NG-MAST STs may be further differentiated by using additional typing methods (125, 180), such as full- or extended-length porB sequencing, PFGE, or Opa typing (9, 19, 58, 69, 83, 96, 115, 153, 172, 174, 180) (Table 1).

NG-MAST has been applied for a number of purposes: for defining gonococcal populations and identifying clusters of infection and particular strains (21, 22, 27, 48–50, 53, 58, 69, 71, 73, 79, 83, 84, 87, 95, 96, 101, 103, 104, 109, 115, 122–125, 128, 138, 153, 155, 156, 162, 165, 172–175, 180, 190–193), to trace sexual contacts (1, 9, 19, 96, 172), for investigating treatment failures (86, 119, 161, 173), and in medical-legal cases (97). NG-MAST has also been evaluated as a tool for predicting specific antimicrobial resistance phenotypes in N. gonorrhoeae isolates (124). However, this application is far from ideal. More research in determining whether certain STs are correlated with specific antibiotic resistance phenotypes and their temporal stability is needed, using a higher number of isolates that are phenotypically, genetically, geographically, and temporally diverse. NG-MAST (http://www.ng-mast.net) is most often used with cultured specimens, and the method must be optimized for potential use in all types of NAAT specimens (191).

(iii) MLST. Multilocus sequence typing (MLST) was first developed and implemented for differentiating Neisseria meningitidis isolates (89) as a general approach for molecular epidemiology and genetic analysis of strain populations. The method is a molecular extension of multilocus enzyme electrophoresis (MLEE) (28, 29, 61, 110, 134, 143), in which electrophoretic mobilities of housekeeping enzymes are analyzed on starch gels. In MLST, the DNA sequences of internal fragments of the alleles of seven or more chromosomal housekeeping genes, which are relatively conserved, slowly evolving, evolutionarily more neutral, and, ideally, distributed throughout the genome, are analyzed. For MLST analysis, different sequences for each locus are assigned divergent allele numbers, and the combination of alleles at the seven loci defines an allelic profile. MLST unambiguously characterizes the sequence type of each isolate. The genetic relatedness of isolates can be presented as a dendrogram constructed by using the matrix of pairwise differences between their allelic profiles (44).

In general and for most microorganisms, the MLST ap-
approach has many important advantages over other typing methods. MLST exhibits a high resolution power (89, 151) provided that the choice and number of examined genes are appropriate (90). Thus, this method is suitable for both epidemiological and population biology studies. MLST analysis is supported by an Internet database (http://www.mlst.net) at which isolate types can be characterized and deposited. There are also some limitations to the MLST approach, which has not yet been extensively used to characterize *N. gonorrhoeae* populations, as identified with other microorganisms. For example, based on MLST analyses, *Salmonella enterica* serovar Typhi (75), *Mycobacterium tuberculosis* (152), and *Vesicularia pestis* (2) have been judged to have clonal population structures. To differentiate such clonal populations by MLST, the loci sequenced should include more rapidly evolving loci (181).

An MLST scheme for typing *N. gonorrhoeae* isolates was developed after examination of the genetic diversity of 18 gonococcal housekeeping genes as potential candidates for a more refined scheme (185). Subsequently, the number and choice of sequenced housekeeping genes have diverged in the few reports involving MLST analysis of *N. gonorrhoeae* isolates (7, 114, 129–131, 163, 185). Some studies have used genes and gene fragments (*n* = 7 [a*b*Z, adk, aroE, fumC, gdh, pdhC, and pgm]) identical to those used for the MLST analysis of *N. meningitidis* isolates (http://pubmlst.org/neisseria; 7, 114), which permits evolutionary studies within the *Neisseria* genus. In some cases, fewer than seven housekeeping genes, combined with more rapidly evolving loci, have been analyzed (81, 82; M. Unemo, unpublished data). However, further evaluation of the specific array of genes useful for *N. gonorrhoeae* population analysis is warranted.

MLST analysis of *N. gonorrhoeae* isolates comprises a high level of reproducibility, typeability, and objectivity. However, the discriminatory ability of the present MLST schemes, examining seven housekeeping loci, is suboptimal for several epidemiological questions involving more microepidemiological analysis (71, 129–131, 163; M. Unemo, unpublished data). An MLST typing scheme which seems to provide a higher discrimination has been developed based on the seven housekeeping genes *a*b*Z, adk, fumC, gdh, gltA, gnd, and pyrD* (184). However, this method needs to be further evaluated. Previous MLST schemes have been used to study the gonococcal population genetic structure, long-term epidemiology, and evolution (7, 114, 129–131, 163, 184, 185). MLST has some significant limitations for routine typing because it is time-consuming and expensive and requires bioinformatics and genetics expertise in order to properly interpret data (Table 1).

**Other Typing Methods**

A number of other molecular typing methods have been used to type gonococcal isolates. Notably, MLEE (the “ancestor” of MLST) indexes allelic variation in multiple chromosomally encoded housekeeping enzymes (28, 29, 61, 110, 134, 143). This method measures relatedness between strains and identifies clonality. MLEE has low discriminatory power if clones are being examined, can be difficult to interpret due to differences in cultural growth conditions and enzyme productivity, and is very laborious. The MLEE method has largely been replaced by MLST for all bacterial species.

Lip typing (166) characterizes the number and sequences of repeats encoding a five-amino-acid sequence (AAEAP) in the *lip* gene, encoding an outer membrane lipoprotein (4, 194). By PCR amplifying *lip* and subsequently differentiating the sizes of PCR products, the number of the repeat-coding sequences can be predicted. DNA sequence analysis of the amplicons further differentiates *N. gonorrhoeae* strains with the same number of repeats, thus subtyping Lip patterns (166). This method has been used to characterize *N. gonorrhoeae* strains in outbreaks of quinolone resistance (105, 106, 167, 168) and in forensic evaluation when combined with other typing methods (30). The discriminatory ability of this method remains to be assessed. Because *lip* is highly conserved in pathogenic *Neisseria* species (4, 194), it may not be possible to differentiate gonococci from other *Neisseria* species in clinical specimens.

Custom-oligonucleotide microarray analysis representing the entire genome of *N. gonorrhoeae* has only been rarely used. However, for research purposes this method has an exceedingly high capacity for assessment of the complete genetic contents of strains and for distinguishing the genetic relatedness between *N. gonorrhoeae* strains as well as strains within the genus *Neisseria* (10, 43a, 112, 113, 147, 148; M. Unemo, unpublished data). Since such methods are expensive and require sophisticated equipment and expertise for interpretation and since results need to be confirmed using PCR and/or ideally DNA sequencing of specific loci, they remain a research tool.

**RECOMMENDED TYPING METHODS FOR SHORT-TERM EPIDEMIOLOGICAL (MICROEPIDEMIOLOGY) QUESTIONS**

The microepidemiological analysis of strains examines the identity of isolates collected during short time periods (days) to a limited number of months or even to a maximum period of a few years. This approach would include typing strains in the following instances: community epidemics; strains in an entire population over a limited time; strains from core groups, larger core groups, or sexual networks; identifying the emergence and transmission of individual (e.g., antimicrobial-resistant) strains; confirmation or discrimination of presumed epidemiological connections in suspected clusters of infection; contact tracing, test of cure, and resolution of medico-legal cases; and characterization of bacterial clones. The best methods for a highly discriminatory, reproducible, typeable, objective, portable, fast, relatively cost-effective, and high-throughput characterization are sequence based, especially full- or extended-length *porB* sequence analysis and NG-MAST (Table 1). The advantages of NG-MAST are that two different highly variable genetic loci (*porB* and *thpB*) are sequenced, which makes it possible to identify recombinational events in one of the loci, and that a publically accessible database (http://www.ng-mast.net) permits interlaboratory comparisons worldwide by assigning numerical allele numbers and sequence types. Both methods can identify clusters of circulating strains, and both identify strains with epidemiological links. NG-MAST presently involves four DNA sequencing reactions, while full- or extended-length *porB* analysis involves only two, making it more cost-effective and less labor-intensive. The main disadvantages of full- or extended-length *porB* sequencing presently include the...
absence of an international database, the assignment of strain types congruent with NG-MAST STs, and the lack of international harmonization on the size of the porB fragment examined. Because the sequences examined in full- or extended-length porB sequencing and NG-MAST are so variable, the analysis should ideally include phylogenetic analysis of the DNA sequences to identify the level of genetic diversity between different sequence types. Furthermore, isolates with identical DNA sequencing types may be further subdivided by using other high-resolution methods, such as PFGE and Opa typing, especially in investigations of extreme microepidemiology. In such cases, an analysis of the exact test and its cost-effectiveness is warranted and should not be routine.

Typing with different highly discriminatory genetic methods on the same N. gonorrhoeae strains generally displays similar levels of discrimination and relatively high congruence; however, there is rarely complete identity (9, 19, 24, 49, 51, 54, 58, 65, 69, 71, 73, 82, 83, 96, 104, 115, 121, 125, 135, 153, 172, 174, 176, 178, 180, 182, 184, 186). This is not surprising since the different genetic targets of the typing methods follow divergent evolutionary pathways and consequently have different evolutionary histories. NG-MAST and full- or extended-length porB sequencing discriminate mostly to the level one requires for identification of a strain within microepidemiological time periods. In contrast, PFGE and Opa typing may also reflect the broader evolution of a strain, and, accordingly, results using these methods need to be interpreted with some caution and using strict interpretative criteria for N. gonorrhoeae (Table 1).

Presently published MLST methods, examining seven housekeeping loci, are not discriminatory enough for analyzing most issues related to microepidemiology. However, an MLST typing method that may provide higher discrimination has been developed (184) and is presently undergoing further evaluation. Finally, auxotyping and serovar determination can still be valuable for primary clinical or epidemiological markers of N. gonorrhoeae. However, given the expense and required expertise in interpretation for these methods, they tend to be used mostly in specific reference laboratories under specific circumstances (Table 1).

**RECOMMENDED TYPING METHODS FOR LONG-TERM OR GLOBAL EPIDEMIOLOGICAL (MACROEPIDEMIOLOGY) QUESTIONS, GONOCOCCAL POPULATION GENETICS, AND EVOLUTION**

For precise and reliable studies dealing with the macroepidemiology (long-term and global epidemiology) of infections caused by N. gonorrhoeae, gonococcal population dynamics over many years or decades, and phylogeny (evolution), sequencing of several more conserved, evolutionarily relatively neutral, and appropriately chosen chromosomal housekeeping genes is crucial. Accordingly, MLST is the method of choice. MLST analysis provides a high level of reproducibility, type-ability, objectivity, and portability.

The methods that are highly suitable for microepidemiological applications, such as NG-MAST and full- or extended-length porB sequencing, examine highly polymorphic and more rapidly evolving genes. Furthermore, the porB gene is evidently subject to a simultaneous evolutionarily positive Darwinian selection for amino acid replacement, purifying selection, and horizontal genetic exchange (11, 52, 65, 130, 131, 137, 146, 163, 176, 178). Notably, in some situations NG-MAST and full- or extended-length porB gene sequencing also may identify global transmission of a single strain over a period of at least 5 years (174). However, in general, NG-MAST and full- or extended-length porB sequence analysis are not ideal for studying macroepidemiological issues involving many years/decades or the global transmission of strains or for precise phylogenetic studies over longer time periods.

**CONCLUSIONS AND FUTURE PERSPECTIVES**

In this review we have described and commented on different methods used for the phenotypic and genotypic typing of N. gonorrhoeae isolates. The performance characteristics of these methods and current recommendations regarding choice of method(s) in divergent situations have also been discussed. Typing of N. gonorrhoeae isolates is crucial for a better understanding of the biology and epidemiology (emergence as well as spread of specific strains) of the organism so that improved public health control measures and preventive interventions might be developed; these include accurate test of cure and contact tracing, identification of core groups, correlation with risk behaviors, and use of effective antimicrobial treatment. There is a need, however, to better harmonize typing methods and their interpretation internationally, coupled with internal and external quality control and quality assurance testing of these methods. Furthermore, there is a need for various regional studies to develop baseline data on strain types so that comparisons of strain emergence and correlation with other epidemiological parameters, such as antibiograms of the isolates, can be made.

In general, DNA sequence-based typing methods are preferred for a variety of reasons: high discriminatory power, reproducibility, rapidity, comparability and transferability of results, identification of previously unknown genetic polymorphisms, study of bacterial population genetics and phylogeny, and high throughput. To obtain the most informative analysis of DNA sequences, it is important not only that raw sequences are examined but also that a more sophisticated analysis, such as a phylogenetic analysis, is undertaken. The sensitivity (i.e., ability to detect small quantities of DNA) of DNA sequence-based methods has the potential to make them useful for direct strain typing of samples obtained noninvasively, such as urine. The disadvantages of DNA sequence-based methods include their relative expense in low-resource settings, their nonoptimization or validation for use on specimens obtained noninvasively and samples with nonviable N. gonorrhoeae (85, 191), and the lack of phenotypic information produced, such as information on immunologically important epitopes which might be valuable for future vaccine development. In a population with a high level of sexual mixing, mixed gonococcal infections, in which an individual is concurrently colonized with more than one strain of N. gonorrhoeae, have been identified through the use of highly discriminatory genotypic techniques (88, 94, 178, 179). However, in examining NAAT specimens, this concurrent colonization may be difficult to demonstrate.

In general, the validity of using any typing method to study
temporal or geographical differences of N. gonorrhoeae, an organism which is nonclonal and highly genetically variable through transformation and which displays a pannemic and sexual population structure (98, 116, 117, 145), should be carefully considered. Data must be interpreted with caution, including the consideration of the time frame. Accordingly, in-depth knowledge is required regarding molecular mechanisms and the time scale of evolutionary changes overall, both of the N. gonorrhoeae genome and of specific genes examined in the different typing methods, in the context of transmission regionally and internationally as well as in different subpopulations.

The advances in genome sequencing technology make it possible to differentiate bacterial strains based on their whole genomes, as has been done with methicillin-resistant Staphylococcus aureus (62) to investigate strain lineage in particular and the time scale of evolutionary changes overall, both of the depth knowledge is required regarding molecular mechanisms and the time scale of evolutionary changes overall, both of the N. gonorrhoeae genome and of specific genes examined in the different typing methods, in the context of transmission regionally and internationally as well as in different subpopulations.

In conclusion, identical methods for typing N. gonorrhoeae isolates should not be used in all situations, i.e., for microepidemiologic, macroepidemiologic, clinical, research, or evolutionary questions. Consequently, the questions asked in relation to the specific situation should guide the use of the most effective typing method or methods. Furthermore, there is no main value or effective (in regard to expenses and labor) use in typing all N. gonorrhoeae isolates if no precise questions exist. Typing results should be interpreted with scientific, clinical, epidemiologic, or other information. We propose that appropriate, validated, and quality-assured DNA sequencing methods should become the methods of choice for typing N. gonorrhoeae isolates worldwide, a recommendation based on all available evidence and the relatively low cost and accessibility of these methods at present.

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REFERENCES


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