MAJOR ARTICLE

Rapid Sequence-Based Identification of Gonococcal Transmission Clusters in a Large Metropolitan Area

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In large metropolitan areas, which typically have the highest rates of gonorrhea, the identification of chains of transmission by use of partner notification is problematic, and there is an increasing interest in applying molecular approaches, which would require new discriminatory high-throughput procedures for recognizing clusters of indistinguishable gonococci, procedures that identify local chains of transmission. Sequencing of internal fragments of 2 highly polymorphic loci, from 436 isolates recovered in London during a 3-month period, identified clusters of antibiotic-resistant and antibiotic-susceptible isolates with indistinguishable genotypes, the vast majority of which were also identical or closely related by other methods, and defined groups of individuals who typically had similar demographic characteristics. This discriminatory sequence-based approach produces unambiguous data that easily can be compared via the Internet and appears to be suitable for the identification of linked cases of gonorrhea and the timely identification of transmission of antibiotic-resistant strains, even within large cities.

Rates of gonococcal infection decreased in many developed countries after the emergence of the AIDS pandemic, but, since the middle of the 1990s, a substantial increase in gonorrhea has been reported in several countries, including the United States and the United Kingdom [1, 2]. It is important that the patterns of disease transmission are understood, to identify and target high-risk groups within communities and to control outbreaks of antibiotic-resistant gonorrhea. Traditionally, partner notification (PN) has been used, but, in large metropolitan areas, which typically have the highest rates of gonorrhea, this approach can be problematic.

Alternatively, molecular typing, used in a timely fashion, could help to identify local outbreaks. The molecular approach assumes that gonococcal isolates from sexual contacts or from a short chain of disease transmission will have indistinguishable genotypes, even when the most discriminatory typing procedures are applied [3–5]. If the typing method indexes genetic variation that accumulates rapidly, such that there are large numbers of distinguishable genotypes circulating within a community, the identification of a cluster of identical gonococcal isolates from individuals within a particular area can be used as an indicator that these individuals may be sexual contacts or part of a short chain of transmission [3]. This type of approach requires careful validation to ensure that clusters of identical gonococcal genotypes typically identify clusters of individuals within chains of transmission. In a recent study, this was achieved by comparing the concordance of clusters defined by molecular typing with those identified by use of demographic data and information obtained through PN [5].

Several different typing procedures have been de-

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veloped for molecular typing of *Neisseria gonorrhoeae* [6–11]. Perhaps the most discriminatory of these procedures is *opa* typing [3], which indexes the genetic variation within the family of 11 highly variable *opa* genes. Typing of *opa* has been shown to be useful for analyzing sexual networks [5, 11], but it is laborious to perform on large numbers of isolates, and results cannot be easily compared between laboratories.

Nucleotide sequence–based typing methods have considerable advantages, because they are high-throughput and provide unambiguous data that readily can be compared between laboratories via the Internet [12, 13]. For epidemiological investigations of transmission of gonorrhea within a community, where the identification of recent chains of transmission is required, a nucleotide sequence–based approach must use genes that are highly variable and within which genetic variation accumulates rapidly [3]. Genetic variation within genes that encode surface-exposed proteins accumulates rapidly as a result of diversifying selection applied by the host immune response, and the nucleotide sequences of such genes should identify large numbers of different sequences (alleles) among isolates of a bacterial pathogen.

The *por* gene encodes the gonococcal outer membrane porin. A typing system based on the complete sequence of this highly variable gene has been evaluated and provides a substantial level of discrimination between isolates [7, 14–16]. However, sequencing the whole *por* gene would be labor intensive, and most of the variation within *por* is likely to be captured by sequencing an internal fragment of the gene, which requires a single sequencing reaction for each DNA strand. The *tbpB* gene encodes the β subunit of the transferrin-binding protein, a surface-exposed peripheral component of the outer membrane that binds the human iron-binding protein transferrin. Studies of *tbpB* sequences from *N. meningitidis* and of the 5 gonococcal *tbpB* sequences available from GenBank have shown extensive sequence variation.

In the present study, we demonstrate that sequencing internal fragments of 2 highly polymorphic antigen-encoding loci, *por* and *tbpB,* provides a high-throughput, unambiguous, typing procedure (*N. gonorrhoeae* multiantigen sequence typing [NG-MAST]) that may be suitable for the identification of individuals within a community who recently have been infected by the same gonococcal strain.

MATERIALS AND METHODS

N. gonorrhoeae *isolates.* Initial evaluation of NG-MAST used a panel of 18 diverse strains that have been used to evaluate typing methods for *N. gonorrhoeae* [6] and 10 pairs of isolates, from known sexual contacts, that each have been shown to be indistinguishable by both phenotypic and genotypic methods. Further evaluation used isolates from the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) in England and Wales [17], which received all gonococci recovered over the course of 3 months in 2000 from 13 different hospitals in London. The MIC to a range of antimicrobials for all strains had been determined as described elsewhere [18]. A total of 195 isolates from the 13 London hospitals (15 from each hospital, selected as every *n*th isolate to cover the 3-month sampling period) were used as a representative subset of the GRASP collection throughout the 3-month time span without any selection by antibiotic susceptibility or by sex of the person from whom the isolate was obtained. All 241 antibiotic-resistant isolates recovered during this surveillance period were also characterized, including an additional 27 isolates within the representative subset that were also resistant, for a total of 268 antibiotic-resistant isolates. These included quinolone-resistant *N. gonorrhoeae* (QRNG; MIC, $\geq$1.0 mg/L), penicillinase-producing *N. gonorrhoeae* (PPNG; β-lactamase positive; tetracycline MIC, <16 mg/L), tetracycline-resistant *N. gonorrhoeae* (TRNG; MIC, $\geq$16 mg/L; β-lactamase negative), PPNG that were also resistant to tetracycline (PP/TRNG; β-lactamase positive; tetracycline MIC, $\geq$16 mg/L), and isolates with reduced susceptibility to ciprofloxacin (MIC, 0.125–0.5 mg/L).

Preparation of DNA. *N. gonorrhoeae* were retrieved from storage at −80°C in glycerol broth, cultured on GC agar base (Becton Dickinson) supplemented with 1% IsoVitaleX (BBL, Becton Dickinson), and incubated overnight at 36°C in 5% CO_2. A single colony was subcultured once before DNA was prepared. In brief, bacterial suspensions (OD at 540 nm of 1.0–2.0; ~10^8–10^9cfu/mL) were made in 0.17 mol/L PBS (pH 7.3; Oxoid), and the bacteria were pelleted by centrifugation at 2000 *g* for 5 min, washed once, resuspended in PBS, and boiled for 5 min. The lysate was centrifuged at 2000 *g* for 5 min, and the supernatant was stored at −20°C.

***Sequencing internal fragments of* por *and* tbpB.** Variable internal regions of *por* and *tbpB* that could be sequenced on both strands by use of a single pair of primers were identified from alignments of the available sequences from GenBank by use of ClustalX software (version 1.8; available at: http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) [19]. Primers for *por* were designed, by use of conserved sequences encoding pre-loop 2 and pre-loop 8 of the porin protein, to amplify both classes of alleles (*por* IA and IB) and amplified a fragment of 737 bp: 5′-^{350}CAA GAA GAC CTC GGC AA^{366}-3′ (*por* forward) and 5′-^{1086}CCG ACA ACC ACT TGG T^{1071}-3′ (*por* reverse) (numbering is based on *por* sequence of strain MS11; GenBank accession number M21289) [20].

Primers for *tbpB* were designed by use of conserved sequences and amplified a fragment of 589 bp: 5′-^{1098}CGT TGT CGG CAG CGC GAA AAC^{1118}-3′ (*tbpB* forward) and 5′-^{1686}TTC ATC GGT GCG CTC GCC TTG^{1666}-3′ (*tbpB* reverse) (numbering is based on *tbpB* sequence from strain UU1008; GenBank ac-

cession number 2286066) [21]. For both *por* and *tbpB,* the length of the amplified fragments varied between isolates because of insertions or deletions in the regions encoding surface-exposed loops.

Sequencing the* por *and* tbpB *fragments. Polymerase chain reaction (PCR) amplification of the *por* gene fragment was performed in reaction volumes of 50 μL, using microtiter plates with a PTC-200 DNA engine (MJ Research). Each PCR contained 50 pmol of each primer, 1× buffer (Qiagen), 2.5 U of *Taq* polymerase (Qiagen), 2 μL of DNA lysate, 0.2 mmol/L each dNTP (Applied Biosystems), and water to a volume of 50 μL. The PCR cycle involved an initial denaturation of 4 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C and cooling to 4°C. PCR amplification of the *tbpB* gene fragment was performed with the *tbpB* primers, by use of the same method, but with an annealing temperature of 69°C.

The amplified DNA fragments were precipitated with 20% polyethylene glycol 8000 and 2.5 mol/L sodium chloride, washed twice in 70% ethanol, dried, and resuspended in sterile water. The DNA fragments were sequenced on each strand with the primers used in the initial PCR amplification by use of an ABI PRISM BigDye Terminator Cycle Sequencing kit (version 2.0; Applied Biosystems) and were applied to an ABI 3700 DNA sequencer. The trace files from the forward and reverse sequencing reactions were analyzed and edited by use of Sequence Navigator software (version 1.01; Applied Biosystems) and trimmed to the correct length.

For *por,* a sequence of 490 bp was used to define the alleles, starting at a conserved sequence and extending from nt 455 in the sequence from strain MS11 [20]. For *tbpB,* a sequence of 390 bp was selected for defining alleles, starting at a conserved sequence and extending from nt 1118 in the sequence from strain UU1008 [21].

Data analysis. The edited and trimmed *por* and *tbpB* sequences were initially compared with each other by use of the Non-Redundant DataBase (available at: http://www.mlst.net), and each different sequence at *por* or *tbpB* was assigned a new allele number in the order that the alleles were discovered. A 2-locus allelic profile was assigned to each isolate by use of the assigned allele numbers at *por* and *tbpB* (e.g., 44–56 has the *por*-44 allele combined with the *tbpB*-56 allele), and each allelic profile was assigned as a sequence type (ST), to provide a convenient descriptor for the strain.

The sequences of the known alleles at *por* and *tbpB* and all known STs were then entered into a publicly accessible database on the NG-MAST Web site (available at: http://www.ng-mast.net). The edited sequences of *por* and *tbpB* from new isolates were subsequently assigned as either known alleles or new alleles, and their STs were assigned by use of the interrogation software available at the NG-MAST Web site.

The expected frequency of an ST was estimated from the product of the frequencies in the database of its *por* and *tbpB* alleles, and the number of isolates of the ST that would be expected in a database by chance was calculated as the product of the expected frequency of the ST and the size of the database. Thus, for an ST that had *por* and *tbpB* alleles that each were present 6 times in a database of 200 isolates, the expected frequency of the ST would be 0.0009 (6/200 × 6/200), and 0.18 (0.0009 × 200) isolates of this ST would be expected in this size of database purely by chance.

Further phenotypic and genotypic characterization of isolates. All isolates were characterized by use of auxotyping [22]. Serotyping was performed by use of the method and nomenclature of Knapp et al. [23], on the basis of the pattern of agglutination using 12 monoclonal antibodies (MAbs). Reproducibility of the antibodies over time is known to be <100% [24], and serovars that differ in only 1 MAb reaction may not be different. Typing of *opa* with *Taq*I was performed by use of the method of O'Rourke et al. [3], and the complex patterns of DNA fragments from the multiple *opa* genes were analyzed by use of Gelcompar software (version 4.1; Applied Maths); isolates were considered to have the same *opa* type only if their fragment patterns were identical. Isolates that differed at ⩽2 fragments may be considered to be closely related in *opa* type.

Calculation of discrimination indices. The discrimination indices (DIs) of the typing methods were calculated by use of Simpson's index of diversity [25].

Reproducibility. The reproducibility of NG-MAST was checked by reamplifying and resequencing 80 GRASP isolates with the original DNA lysates. For 20 GRASP isolates, new DNA lysates were prepared.

RESULTS

Sequence diversity within the* por *and* tbpB *fragments. The primers amplified the internal fragments of *por* and *tbpB* from all of the isolates, and sequences on both strands were obtained from 474 gonococcal isolates. There were 166 different *por* alleles and 86 different *tbpB* sequences, resulting in 197 different 2-locus allelic profiles. Each allelic profile was assigned a different ST number. The correctly trimmed sequences of all known *por* and *tbpB* alleles are available at the NG-MAST Web site (http://www.ng-mast.net).

Among the 474 isolates, the most common alleles were *por*-2 and *tbpB*-16, which were present in 27 and 62 isolates, respectively. The most common allelic profile (2–16) was ST2, which possessed the most common alleles at each locus, and was represented by 22 isolates. The 10 most prevalent *por* alleles (present in ⩾10 isolates) were, in all cases, associated with the most prevalent *tbpB* alleles. These values are distorted by the presence of large clusters of indistinguishable isolates among

the antibiotic-resistant isolates that were characterized (see below). Within the representative set of 195 isolates from GRASP, which provides an unbiased measure of the allelic and ST diversity among isolates recovered in London over the course of a 3-month period, there were 57 *por* alleles and 23 *tbpB* alleles that were unique to 1 isolate (figure 1*A*). The largest ST cluster included 10 isolates, and there were 14 ST clusters that included 2 isolates each (figure 1*B*)

Initial evaluation of the NG-MAST procedure. In the panel of 18 strains that had different *opa* types [6], each had a different *por* allele, and there were 17 different *tbpB* alleles; thus, all 18 strains had a different ST. Ten pairs of isolates from 10 couples who each named the other as their sexual contact were characterized, and each pair of isolates had identical STs, whereas the different pairs all had distinct STs.

The NG-MAST procedure was reproducible, as expected for a sequence-based approach. The same STs were obtained from a set of 80 previously characterized GRASP isolates by use of the original DNA lysates, and, for 20 GRASP isolates for which new DNA lysates were prepared, the STs assigned were the same as those obtained from the initial lysates.

Clustering among representative isolates from GRASP 2000. Among the representative 195 isolates, there were 88 *por* alleles, 39 *tbpB* alleles, and 103 different STs, of which 69 STs were represented by a single isolate. The largest cluster (ST24 [allelic profile 17–16]) included 10 isolates, and single clusters of 9 and 7 isolates each were also present. There were 3 clusters of 6 isolates, 4 clusters of 5 isolates, 4 clusters of 4 isolates, 6 clusters of 3 isolates, and 14 clusters of 2 isolates. Isolates of a given ST were typically identical or closely related, with regard to *opa* type, antibiotic-resistance profile, and auxotype/serovar (AS; phenotype), and were concordant with the available demographic data. Table 1 shows the concordance for the 14 clusters that included at least 4 isolates. Of the 14 ST clusters, 6 were concordant by indistinguishable *opa* profiles, 12 were concordant when up to 2 DNA fragment differences in the *opa* profile were allowed, and 13 were concordant when up to 3 DNA fragment differences in the *opa* profile were

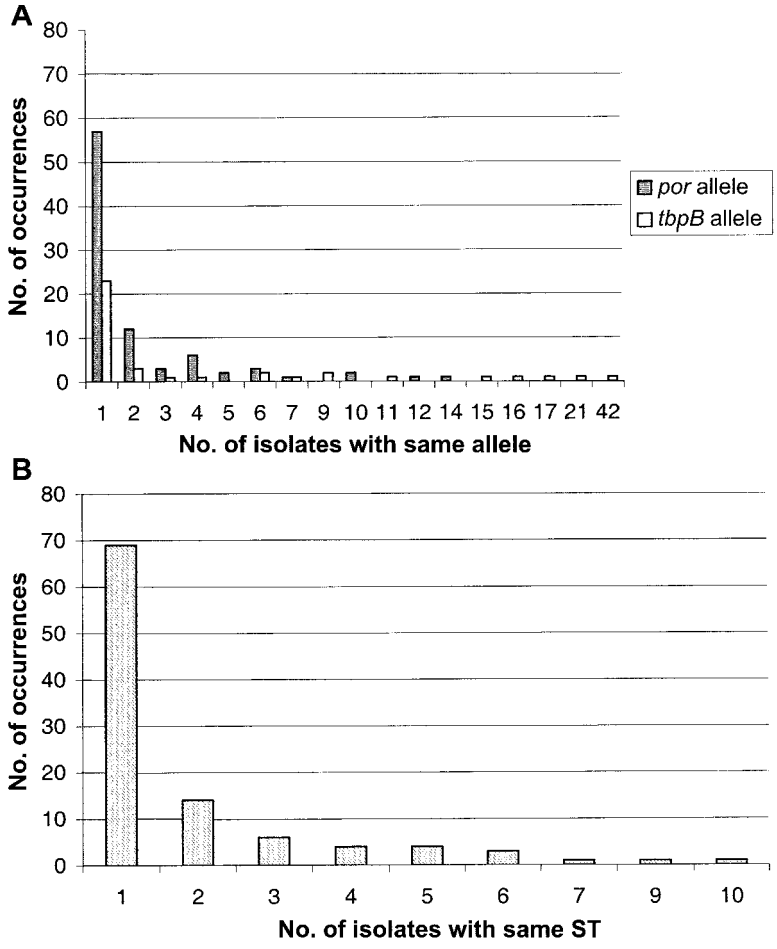

Figure 1. No. of occurrences of different *por* and *tbpB* allele cluster sizes *(A)* and different sequence type (ST) cluster sizes *(B),* among the 195 representative isolates from the Gonococcal Resistance to Antimicrobials Surveillance Programme.

Table 1. Concordance between genotypic, phenotypic, and demographic data within the major clusters of isolates of the same sequence type (ST) from the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP).

				Phenotypic data			Demographic data					
ST (*por* allele–*tbpB* allele)	No. of isolates	Expected frequency of ST[a]	Expected no. of isolates of ST[a]	Auxotype and serovar concordance[b]	MIC concordance[c]	*opa* type concordance[d]	Female, no. of subjects	Male, no. of subjects	Sexuality	Area of residence in London	Ethnic group	No. of patients who reported sexual contact abroad within the previous 3 months
2 (2–16)	9	0.015	2.925	X	X (8/9)	X (5/9; 4/9 [2])	5	4	Hetero	E (4), S (1), N (2), NK (2)	B (3), NK (6)	1
3 (2–5)	5	0.008	1.560	X	X	X	3	2	Hetero	S (4), NK (1)	B (4) NK (1)	1
4 (3–4)	4	0.002	0.390	X	X	X (3/4; 1/4 [2])	0	4	MSM	N (1), E (1), We (1), NK(1)	Wh (3), B (1)	0
6 (5–16)	4	0.004	0.780	X (1)	X	X	2	2	Hetero	S (3), We (1)	Wh (2), B (1), NK (1)	0
8 (7–3)	7	0.005	0.975	X	X	X (4/7; 2/7 [1]; 1/7 [2])	4	3	Hetero	S (3), N (2), NK (2)	B (5), NK (2)	0
12 (8–5)	5	0.003	0.585	X (1, 2)	X	X (3/5; 1/5 [1]; 1/5 [2])	1	4	Hetero	S (3), N (2)	B (3), NK (2)	0
13 (9–3)	6	0.003	0.585	X (1)	X	X	2	4	Hetero	S (2), E (1), N (1), NK (2)	Wh (3), B (1), A (1), NK (1)	0
20 (13–4)	5	0.002	0.390	X (1)	X	X	1	4	Hetero (2), MSM(3)	N (1), E (1), S (1), We (1), NK (1)	Wh (4), NK (1)	0
24 (17–16)	10	0.011	2.145	X (1)	X	X (7/10; 3/10 [3])	7	3	Hetero	N (5), S (3), E (1), Birmingham (1)	B (5), Wh (4), NK (1)	0
26 (19–24)	4	0.003	0.585	X	X	X	0	4	Hetero (2), MSM(2)	N (3), S (1)	Wh (3), B (1)	0
38 (28–19)	4	0.0006	0.117	X	X	X (3/4; 1/4 [2])	2	2	Hetero	S (2), We (1), Greater London (1)	B (3), Wh (1)	0
40 (30–35)	6	0.001	0.195	X	X	X	0	6	MSM	S (3), NK (3)	Wh (3), NK (3)	0
44 (33–5)	6	0.005	0.975	X (1)	X	X (4/6; 2/6 [1])	4	2	Hetero	N (3), S (2), E (1)	B (5), W (1)	1
51 (39–27)	5	0.001	0.195	X	X	X (3/5; 2/5 [5])	3	2	Hetero	N (1), S (1), Greater London (3)	Wh (3), B (2)	0

NOTE. A, Asian; B, black (including black Caribbean and black African); E, east; Greater London, outskirts of London; hetero, heterosexual; MSM, men who have sex with men; N, north; NK, not known; S, south; We, west; Wh, white (including white British, white Irish, and white other).

[a] The expected frequency of each ST was calculated from the frequencies of the *por* and *tbpB* alleles among the 195 representative isolates from GRASP 2000. The expected no. of isolates was calculated from the expected frequency times 195.

[b] The "X" indicates that all isolates of the ST were concordant in both auxotype and serovar. Isolates within 5 STs varied by 1 monoclonal antibody (MAb) reaction (indicated by the nos. in parentheses); in 1 case (ST 12) there was an isolate that differed by 1 MAb reaction and 2 that differed by 2 MAb reactions (the latter were classed as nonconcordant in serovar)

[c] The "X" indicates that the MICs of isolates to penicillin, ciprofloxacin, tetracycline were concordant. Unless stated, all isolates were concordant in the cluster.

[d] The "X" indicates that all isolates of the ST had an identical *opa* type. Where isolates of an ST had different *opa* types, the proportion of isolates with each *opa* type is shown in parentheses, and the no. of differences in the DNA fragment patterns of the minority *opa* type(s) is in brackets.

allowed. Isolates of ST51 (39–27) resolved into 2 groups that had 5-band differences in their *opa* profiles, although they were concordant in phenotype. Good concordance was also obtained for most of the smaller clusters. For example, 11 of the 14 ST pairs showed complete concordance in phenotype and *opa* type.

Comparison of NG-MAST and* opa *typing. The 195 representative isolates from GRASP were all characterized by both NG-MAST and *opa* typing, which gave highly congruent results, with all isolates of the same *opa* type having the same ST. However, for 25 isolates, *opa* typing resolved isolates assigned to the same ST: 5 differed in their *opa* type by >2 DNA fragments from the other isolate(s) of the same ST. Thus, 80% of isolates of the same ST that differed in *opa* type were judged to be very similar in genotype, because the complex DNA fragment patterns from the *opa* genes differed only slightly (DI for NG-MAST, 0.99; DI for *opa* typing, 0.99 [both DI values were calculated by use of Simpson's index of diversity]).

Clusters among high-level antibiotic-resistant isolates. A total of 19 isolates were QRNG and were resolved into 15 STs, 12 of which were represented by a single isolate. There were 2 pairs and a group of 3 isolates with the same ST, and the isolates within each ST had identical (1 pair) or very closely related phenotypes (isolates within the other pair and the group of 3 differed by 1 MAb reaction). There were 14 different A/S classes among the 19 isolates, consistent with the molecular data, which suggested a relatively diverse group. Six of the individuals acquired their infection abroad rather than through endemic transmission within London.

A total of 47 isolates had reduced susceptibility to ciprofloxacin and were resolved into 15 STs, 8 of which included 1 isolate; each of these was of a different A/S class. There was a large cluster of 13 isolates (ST66 [alleleic profile 52–33]) that had the same auxotype (NR) and identical or very closely related serovars (IB-1 or IB-2, which differ by 1 reaction difference) and had reduced susceptibility to penicillin (MIC, 0.125–0.5 mg/L). Ten of the 13 isolates were from men who have sex with men (MSM) (MSM status was unknown for the other 3 men), none of whom reported having sexual contacts abroad, and 8 of whom lived in East or South London.

Sixty-two isolates were PPNGs and were resolved into 20 STs, 10 of which included 1 isolate. Nineteen isolates were ST73 (59–23); all isolates in this cluster had the same penicillinase plasmid type (3.2 mDa), were susceptible to ciprofloxacin, were auxotype NR/P, and were serovar IB-4. Eighteen of the 19 isolates were from heterosexual subjects living in South or West London who reported no sexual contacts abroad.

Eighty-eight isolates were TRNG and were resolved into 28 different STs, 13 of which included a single isolate. There were 2 large clusters of 14 isolates each (ST2 [allelic profile 2–16] and ST15 [allelic profile 108–19]). The isolates of ST2 all had the US (large) type of tetracycline-resistance plasmid, were auxotype NR/PA, were serovar IB-11 in 12 isolates and NR/IB-17 in the other 2 isolates (3 reaction differences), were susceptible to ciprofloxacin, and had reduced susceptibility to penicillin. These isolates were from heterosexual subjects predominantly living in East London who reported no sexual contacts abroad. The isolates of ST15 also had the US (large) type of plasmid, were auxotype NR, were serovar IB-17 or IB-23 (1 reaction difference), were susceptible to ciprofloxacin, had reduced susceptibility to penicillin, and were from heterosexual subjects predominantly living in South London (10/14), only 1 of whom reported sexual contact abroad.

Fifty-two isolates were PP/TRNG and were resolved into 19 different STs, of which 14 included a single isolate. There were 2 large clusters of 17 and 13 isolates each. The cluster of 17 isolates (ST13 [allelic profile 9–3]) had the Dutch (small) tetracycline-resistance plasmid and the 3.2-mDa penicillinase plasmid, were susceptible to ciprofloxacin, and had closely related A/S classes. The isolates were all from heterosexual subjects, 10 of whom lived in South London and 1 of whom reported a sexual contact abroad. The cluster of 13 isolates (ST130 [allelic profile 121–21]) had the US (large) tetracycline-resistance plasmid, the 3.2-mDa penicillinase plasmid, were susceptible to ciprofloxacin, had very closely related A/S classes, and were from heterosexual subjects living in South London (11/13), 1 of whom reported a sexual contact abroad. A summary of the results, by number of different STs and the largest ST cluster for each groups of strains used to evaluate NG-MAST, is shown in table 2.

DISCUSSION

The NG-MAST procedure provides a new and promising nucleotide sequence–based approach to rapid but precise characterization of gonococcal isolates. The method produces unambiguous data that greatly simplify the identification of clusters of indistinguishable isolates within a local community or the recognition of an outbreak caused by the same strain in different communities. NG-MAST is a simpler procedure than *opa* typing and is much more readily applicable to the characterization of very large collections of gonococci.

Variation within the *por* and *tbpB* loci is likely to be selected by the human immune response, and, as expected, there were large numbers of alleles at both loci, even though the isolates were almost all collected from subjects in a single city over the course of 3 months. Importation of new strains into the community, the generation of new alleles by mutation, and shuffling of the many *por* and *tbpB* alleles by recombination during mixed gonococcal infections [26] should lead to new combinations of alleles at these 2 loci and a rapidly changing population of STs.

There were almost twice as many alleles at *por* (166) as at

Table 2. Strains used in evaluation of *Neisseria gonorrhoeae* multiantigen sequence typing and the no. of different sequence types (STs), by strain group and the largest ST cluster size.

Strain group	No. of strains	No. of different STs	Largest ST cluster
Representative sample from GRASP 2000	195	103	10
Resistant isolates from GRASP 2000[a]	268	76	19
QRNG	19	15	3
Reduced susceptibility to ciprofloxacin[b]	47	15	13
PPNG	62	20	19
TRNG	88	28	14
PP/TRNG	52	19	17
All GRASP isolates	436	169	22
Panel isolates[c]	18	18	1
10 Pairs from sexual contacts	20	10	2
Total isolates	474	197	22

NOTE. GRASP, Gonococcal Resistance to Antimicrobials Surveillance Programme; PPNG; penicillinase-producing *N. gonorrhoeae;* PP/TRNG, PPNG that were also resistant to tetracycline; QRNG, quinolone-resistant *N. gonorrhoeae;* TRNG, tetracycline-resistant *N. gonorrhoeae.*

[a] Within the 195 representative sample of isolates from GRASP 2000, there were 27 isolates that had resistance to penicillin, tetracycline, or ciprofloxacin (high-level or reduced susceptibility). All of the additional 241 antibiotic-resistant isolates from GRASP 2000 were also characterized, for a total of 268 antibiotic-resistant isolates.

[b] MIC, 0.125–0.5 mg/L.

[c] A panel of 18 diverse gonococcal isolates used to evaluate the discriminatory power of gonococcal typing scheme.

tbpB (86), and the clustering achieved with *por* alone was not very different from that obtained with both loci. Thus, the 474 isolates were resolved into 166 genotypes on the basis of differences in the *por* fragment, and the addition of the second locus increased this number to only 197. Among the representative sample of 195 isolates from London hospitals over the course of 3 months, there were 88 *por* alleles, which increased to 103 distinguishable genotypes when both loci were used.

The relatively small increase in resolution obtained by use of the second locus implies that most isolates possessing the same *por* allele were likely to be the same strain, because they also possessed the same *tbpB* allele. However, the addition of *tbpB* was important, because some strains may be wrongly grouped by *por* alone (i.e., as a result of the horizontal spread of the same *por* allele into different strains), and the presence of nearly 100 *tbpB* alleles provided a further level of resolution. The limited increase in resolution by the use of a second locus suggests that the addition of a third polymorphic gene is unnecessary and would lead to little further resolution.

There was more clustering among the antibiotic-resistant isolates than among the representative isolates ($P < .0001$, χ^2 test), and, among the 474 isolates that were characterized, the allele frequencies and extent of clustering were biased by the inclusion of all high-level antibiotic-resistant strains. The 195 representative isolates provided an unbiased measure of the extent of clustering among isolates recovered in London during GRASP 2000. There were 14 clusters of ≥ 4 isolates, and those with the same ST typically had identical or closely related *opa* types and phenotypes. For 11 of these 14 clusters, the expected frequency of the ST was ≤ 0.005, and <1 isolate of these STs would be expected by chance in a database of 195 isolates, which adds support to the view that the larger clusters are likely to be valid.

The clusters of isolates indistinguishable by NG-MAST typically appear to represent multiple occurrences of the same strain in the community and the phenotypic data, and demographic features support the view that these are valid clusters. For antibiotic-resistant isolates, the extent of clustering may also indicate whether there is local transmission (large clusters) or repeated importation from abroad (little clustering), both of which were evident among these isolates.

These results strongly suggest that the appearance of a cluster of isolates of the same ST in a city will generally be indicative of an outbreak and can be used to identify a group of individuals for whom control measures can be targeted. However, prospective studies combining NG-MAST with PN are required to rigorously establish whether individuals in a given city who share isolates of the same ST are part of the same sexual network.

NG-MAST lacks the very high level of resolution of *opa* typing, and there are indications that some clusters of isolates of the same ST can be resolved into subclusters (e.g., ST51 [allelic profile 39–27] was resolved by *opa* typing into 2 sub-

clusters whose profiles differed by 5 DNA fragments). However, in the majority of cases, NG-MAST appears to have sufficient resolution to identify valid clusters, and the procedure has overwhelming advantages for studies of gonorrhea in large metropolitan areas since, unlike *opa* typing, it could be used to characterize, in a timely manner, all gonococcal isolates recovered at multiple clinics across a large city. Only 4 sequencing reactions are required per isolate, much of the procedure can be automated [27], and results can be obtained within a week of receiving samples from patients diagnosed with gonorrhea.

The NG-MAST Web site (http://www.ng-mast.net), which is currently hosted by Imperial College London, maintains the central database that includes the sequences of all known alleles at each locus and details of the known STs. Investigators can assign their alleles and STs by interrogating the Web site and can obtain allele numbers and ST numbers for new alleles or strains, which then become available to others via the public database. In this way, isolates recovered in 1 city can be compared with those from other cities or other countries. The ability to compare gonococcal isolates from different cities or countries may be less important for gonorrhea than for many other diseases [3], but is likely to be useful in the case of antibiotic-resistant strains that may be imported into several countries from a common source. For local public health purposes, results must be available in a timely fashion to identify clusters and to target prevention efforts. This can be achieved by use of NG-MAST.

An increase in the incidence of gonococcal infections in many countries is occurring despite the availability of genitourinary medicine clinics that often have free access to treatment for patients and interventions targeted at specific age, ethnic, and high-risk groups. Effective antimicrobials are available for treatment, although the increasing levels of antibiotic resistance are reducing the effectiveness of these therapies. We believe that a new approach to controlling gonorrhea is required and that the use of NG-MAST may be the tool to provide this. The high-throughput of NG-MAST could be used to identify endemic transmission clusters within a defined area and to target PN to individuals who are likely to represent high-risk groups. The outcome of the targeted PN could be monitored by the persistence or removal of the outbreak strains and could be particularly effective in limiting the transmission of antibiotic-resistant strains in the community.

GONOCOCCAL RESISTANCE TO ANTIMICROBIALS SURVEILLANCE PROGRAMME STUDY MEMBERS

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References

1. Public Health Laboratory Service, Department of Health and Social Services and Public Safety, and the Scottish Information and Statistics Division 5 Collaborative Group. Sexually transmitted infections in the UK: new episodes seen at genitourinary medicine clinics, 1991 to 2001. London: Public Health Laboratory Service, **2002**.
2. Centers for Disease Control and Prevention (CDC). Tracking the hidden epidemics: trends in STDs in the United States. Atlanta: CDC, **2000**.

3. O'Rourke M, Ison CA, Renton AM, Spratt BG. Opa-typing: a high-resolution tool for studying the epidemiology of gonorrhea. Mol Microbiol **1995**; 17:865–75.
4. Ison CA. Genotyping of *N. gonorrhoeae.* Curr Opin Infect Dis **1998**; 11:43–6.
5. Ward H, Ison CA, Day SE, et al. A prospective social and molecular investigation of gonococcal transmission. Lancet **2000**; 356:1812–7.
6. Van Looveren M, Ison CA, Ieven M, et al. Evaluation of the discriminatory power of typing methods for *Neisseria gonorrhoeae.* J Clin Microbiol **1999**; 37:2183–8.
7. Viscidi RP, Demma JC, Gu J, Zenilman J. Comparison of sequencing of the *por* gene and typing of the *opa* gene for discrimination of *Neisseria gonorrhoeae* strains from sexual contacts. J Clin Microbiol **2000**; 38: 4430–8.
8. Thompson DK, Deal CD, Ison CA, Zenilman JM, Bash MC. A typing system for *Neisseria gonorrhoeae* based on biotinylated oligonucleotide probes to PIB gene variable regions. J Infect Dis **2000**; 181:1652–60.
9. Trees DL, Schulz AJ, Knapp JS. Use of the neisserial lipoprotein (Lip) for subtyping *Neisseria gonorrhoeae.* J Clin Microbiol **2000**; 38:2914–6.
10. Viscidi RP, Demma JC. Genetic diversity of *Neisseria gonorrhoeae* housekeeping genes. J Clin Microbiol **2003**; 41:197–204.
11. Palmer HM, Leming JP, Turner A. Investigation of an outbreak of ciprofloxacin-resistant *Neisseria gonorrhoeae* using a simplified opa-typing method. Epidemiol Infect **2001**; 126:219–24.
12. Maiden MC, Bygraves JA, Feil E, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA **1998**; 95:3140–5.
13. Spratt BG. Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the Internet. Curr Opin Microbiol **1999**; 2:312–6.
14. Cooke SJ, de la Paz H, La Poh C, Ison CA, Heckels JE. Variation within serovars of *Neisseria gonorrhoeae* detected by structural analysis of outer-membrane protein PIB and by pulsed-field gel electrophoresis. Microbiology **1997**; 143:1415–22.
15. Hobbs MM, Alcorn TM, Davis RH, et al. Molecular typing of *Neisseria gonorrhoeae* causing repeated infections: evolution of porin during passage within a community. J Infect Dis **1999**; 179:371–81.
16. Unemo M, Olcen P, Berglund T, Albert J, Fredlund H. Molecular epidemiology of *Neisseria gonorrhoeae:* sequence analysis of the *porB* gene confirms presence of two circulating strains. J Clin Microbiol **2002**; 40: 3741–9.
17. Paine TC, Fenton KA, Herring A, et al. GRASP: a new national sentinel surveillance initiative for monitoring gonococcal antimicrobial resistance in England and Wales. Sex Transm Infect **2001**; 77:398–401.
18. Ison CA, Martin IM. Gonorrhoea in London: usefulness of first line therapies. London Gonococcal Working group. Sex Transm Infect **2002**; 78:106–9.
19. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **1997**; 24: 4876–82.
20. Carbonetti NH, Simnad VI, Seifert HS, So M, Sparling PF. Genetics of protein I of *Neisseria gonorrhoeae:* construction of hybrid porins. Proc Natl Acad Sci USA **1988**; 85:6841–5 (erratum: Proc Natl Acad Sci USA 1989; 86:1317).
21. Cornelissen CN, Anderson JE, Sparling PF. Characterization of the diversity and the transferrin-binding domain of gonococcal transferrin-binding protein 2. Infect Immun **1997**; 65:822–8.
22. Copley CG, Egglestone SI. Auxotyping of *Neisseria gonorrhoeae* isolated in the United Kingdom. J Med Microbiol **1983**; 16:295–302.
23. Knapp JS, Tam MR, Nowinski RC, Holmes KK, Sandstrom EG. Serological classification of *Neisseria gonorrheae* with use of monoclonal antibodies to gonococcal outer membrane protein I. J Infect Dis **1984**; 150:44–8.
24. Gill MJ. Serotyping *Neisseria gonorrhoeae:* a report of the Fourth International workshop. Genitourin Med **1991**; 67:53–7.
25. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol **1988**; 26:2465–6.
26. Martin IM, Ison CA. Detection of mixed infection of *Neisseria gonorrhoeae.* Sex Transm Infect **2003**; 79:56–8.
27. Jefferies J, Clarke SC, Diggle MA, Smith A, Dowson C, Mitchell T. Automated pneumococcal MLST using liquid-handling robotics and a capillary DNA sequencer. Mol Biotechnol **2003**; 24:303–8.