Evaluation of *Neisseria gonorrhoeae* Multiple-Locus Variable-Number Tandem-Repeat Analysis, *N. gonorrhoeae* Multiantigen Sequence Typing, and Full-Length *porB* Gene Sequence Analysis for Molecular Epidemiological Typing

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The performance characteristics of *Neisseria gonorrhoeae* multilocus variable-number tandem-repeat analysis were evaluated, by comparison with *N. gonorrhoeae* multiantigen sequence typing and full-length *porB* sequence typing. Assessment of the relatedness of intra- and interpatient isolates showed that all three genotyping techniques display a high resolution and typeability.

The continuing high incidence of gonorrhea in less-resourced settings and increasing incidence in many industrialized countries since the mid- or late 1990s demand more efficient national and international surveillance of *Neisseria gonorrhoeae* infection. Furthermore, the absence of an effective vaccine and worldwide spread of multidrug-resistant *N. gonorrhoeae* strains, which may result in untreatable gonorrhea in certain circumstances, substantially add to this demand (5–7, 21, 26). Typing for differentiation of *N. gonorrhoeae* strains can provide valuable data for epidemiological surveillance. Traditionally, phenotypic typing schemes were used that often lacked sufficient discriminatory power. Therefore, objective, precise, robust, and highly discriminatory molecular typing techniques have become crucial tools that, combined with epidemiological data, can be used to elucidate *N. gonorrhoeae* transmission networks and core groups (21). Many molecular typing techniques for *N. gonorrhoeae* have been described in recent decades (1, 4, 9–11, 13, 16, 17, 20, 21, 26, 27). At present, the most frequently used genotyping method is *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), which is based on DNA sequence analysis of more variable internal regions of two highly polymorphic loci, *porB* (490 bp are analyzed) and *tbpB* (390 bp analyzed) (13, 21). NG-MAST is a robust technique with high discriminatory power and reproducibility and gives access to a global online database (www.ng-mast.net). Another powerful, discriminative genotyping technique is *porB* gene sequencing, which uses the full-length or a large part of the *porB* gene as a single polymorphic locus (12, 21, 25, 26). An alternative DNA typing technique, which has been successfully applied for the analysis of several bacterial pathogens, makes use of the variation in the number of tandem repeats in different loci dispersed over the genome, i.e., multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) (see also www.mlva.net) (18, 29). Recently, an *N. gonorrhoeae* MLVA (NG-MLVA) was developed and evaluated clinically and proven to provide high-resolution and cost-effective genotyping by targeting five polymorphic repeat loci (8). However, this NG-MLVA has not been compared to any of the other previously validated, effective, and frequently used *N. gonorrhoeae* genotyping methods.

In the present study, the performance characteristics of this

recently developed NG-MLVA were compared to the internationally frequently used NG-MAST and full-length *porB* sequence typing.

During 1 year (2002 to 2003), *N. gonorrhoeae* isolates ($n = 885$) were obtained from 696 patients that visited the Amsterdam outpatient sexually transmitted infection (STI) clinic in the Netherlands (8, 10). We selected for the present study (i) the isolates of 118 patients ($n = 252$) that were diagnosed at one visit as infected with *N. gonorrhoeae* at two or three anatomical locations and (ii) the isolates of 54 couples ($n = 108$), being self-reported sexual partners. Since some of the sexual partners had *N. gonorrhoeae* infections at multiple anatomical locations, their isolates belonged to both of these groups. Hence, a total of 329 rather than 360 *N. gonorrhoeae* isolates were genotyped.

To challenge the stability of the genotyping methods, we also examined a panel that consisted of three different strains from three divergent verified sexual transmission chains (in total six isolates from six patients) and the pre- and posttreatment isolates from three cases of treatment failures using extended-spectrum cephalosporins (in total six isolates) (23, 24). Finally, the 2008 WHO *N. gonorrhoeae* reference strains were included as quality controls (22).

All isolates were assigned an NG-MLVA type that consisted of a string of five integers derived from the number of repeats in each of the five VNTR loci (VNTR04-03, VNTR04-10, VNTR07-02, VNTR15-02, and VNTR16-01) as described previously (8). All isolates were also genotyped using NG-MAST and by full-length *porB* sequencing as described previously (13, 28). Accordingly, each isolate was assigned an NG-MAST sequence type (ST) and an arbitrarily defined *porB* ST. The locations of the different geno-

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FIG 1 Locations of the genotyping loci in the genome of *N. gonorrhoeae* FA1090 (GenBank accession no. [AE004969.1](#)).

typing loci in the genome of *N. gonorrhoeae* FA1090 (GenBank accession no. [AE004969.1](#)) are illustrated in Fig. 1.

The results of NG-MLVA versus NG-MAST and full-length *porB* sequencing of all 329 STI clinic isolates from patients positive at multiple anatomical locations and from the sexual partners within couples are presented in Tables 1 and 2.

The congruence between the typing techniques was determined by comparing the *N. gonorrhoeae* genotypes at two (102 patients) or three (16 patients) anatomical sites in the same patient, totaling 118 patients. According to NG-MLVA, NG-MAST, and *porB* sequencing, 77 (65.3%), 97 (82.2%), and 94 (79.7%) patients, respectively, had indistinguishable *N. gonorrhoeae* isolates at all anatomical locations (Table 1). When NG-MLVA types having a single-locus variant (SLV) were also interpreted as identical, 102 (86.4%) patients had indistinguishable isolates at all anatomical locations (Table 1). In that case, in 90 (88.2%) of these 102 patients, both NG-MLVA and NG-MAST identified all isolates from different anatomical sites as indistinguishable. The same identity was seen for NG-MLVA and *porB* sequencing in 87 (85.3%) patients, and for all three typing methods identity was seen in 82 (80.4%) patients (Table 2). In eight out of nine (88.9%) patients with *N. gonorrhoeae* isolates that contained multilocus variants (MLV), nonidentical NG-MAST sequence types (STs) and *porB* sequences were also identified.

NG-MAST and *porB* sequencing further differentiated the NG-MLVA types of 12 (11.8%) and 15 (14.7%) patients, respec-

tively, that had indistinguishable NG-MLVA types (i.e., one SLV allowed; $n = 102$) at different anatomical locations (Table 2). In total, NG-MLVA, NG-MAST, and *porB* sequencing provided concordant clustering for 90 (76.3%) patients, which is a relatively high proportion.

The congruence between the identified genotypes of each typing technique and the identified partner links was also examined. Applied to the 54 couples, NG-MLVA, NG-MAST, and *porB* sequencing identified 40 (74.1%), 44 (81.5%), and 47 (87.0%) of the couples as having indistinguishable *N. gonorrhoeae* isolates, respectively. When NG-MLVA types having an SLV were considered as identical, the sexual partners in 47 (87.0%) of the couples had indistinguishable isolates (Table 1). Using this interpretation of NG-MLVA, we identified that NG-MLVA and NG-MAST provided concordant clustering for 41 (87.2%) of these 47 couples, NG-MLVA and *porB* sequencing for 44 (93.6%) couples, and NG-MLVA and both NG-MAST and *porB* sequencing for 39 (83.0%) couples (Table 2). The sexual partners in four couples had isolates with an MLV, and these isolates, within the couples, also contained nonidentical NG-MAST STs and *porB* sequences (Table 2).

Six (12.8%) and three (6.4%) couples with identical NG-MLVA types (i.e., one SLV allowed; $n = 47$ couples) were further differentiated by NG-MAST and *porB* sequencing, respectively (Table 2). In total, NG-MLVA, NG-MAST, and *porB* sequencing provided fully concordant clustering for 43 (79.6%) couples.

The isolates of the three divergent *N. gonorrhoeae* strains from three sexual transmission chains with confirmed epidemiological links (isolates sampled 1 week, 4 months, and 9 months apart) had NG-MLVA types that were identified as identical (no locus variant [NLV]), SLV, and MLV, respectively. The SLV had a polymorphism in VNTR07-02, whereas the MLV had polymorphisms in four out of five VNTR loci, with the VNTR16-01 remaining the only stable locus. NG-MAST and *porB* sequencing identified both isolates in each transmission chain as indistinguishable, indicating the genetic stability of the *porB* and *tbpB* loci.

In addition, the pre- and posttreatment *N. gonorrhoeae* isolates ($n = 6$) of two cases with cefixime treatment failure with 10 and 20 days between sampling, respectively, and one case with ceftriaxone treatment failure (pharyngeal infection) with 2 months between sampling were also typed. The NG-MLVA types, NG-

TABLE 1 Identical genotypes in sets of *N. gonorrhoeae* isolates obtained from different anatomical sites of the same patient and from couples using NG-MLVA, NG-MAST, and full-length *porB* sequencing

Typing method ^a	No. (%) of patients or couples with sets of isolates with an identical genotype	
	Patients ($n = 118$)	Couples ($n = 54$)
NG-MLVA (NLV)	77 (65.3)	40 (74.1)
NG-MLVA (NLV+SLV)	102 (86.4)	47 (87.0)
NG-MAST	97 (82.2)	44 (81.5)
<i>porB</i> sequencing	94 (79.7)	47 (87.0)

^a NG-MLVA, *N. gonorrhoeae* multiple-locus variable-number tandem-repeat analysis; NLV, no locus variant; SLV, single-locus variant; NG-MAST, *N. gonorrhoeae* multiantigen sequence typing.

TABLE 2 Comparison of NG-MLVA with NG-MAST and full-length *porB* sequencing using sets of *N. gonorrhoeae* isolates obtained from different anatomical sites of 118 patients and from 54 couples^a

Group	NG-MLVA profiles	No. of patients or couples ^b	No. (%) of patients or couples ^c					
			NG-MAST sequence types		<i>porB</i> sequence types		NG-MAST and <i>porB</i> sequence types	
			Identical	Nonidentical	Identical	Nonidentical	Identical	Nonidentical
Patients	NLV + SLV	102	90 (88.2)	12 (11.8)	87 (85.3)	15 (14.7)	82 (80.4)	7 (6.9)
	DLV	7	6 (85.7)	1 (14.3)	6 (85.7)	1 (14.3)	6 (85.7)	1 (14.3)
	MLV	9	1 (11.1)	8 (88.9)	1 (11.1)	8 (88.9)	1 (11.1)	8 (88.9)
Couples	NLV + SLV	47	41 (87.2)	6 (12.8)	44 (93.6)	3 (6.4)	39 (83.0)	1 (2.1)
	DLV	3	3 (100)	0	3 (100)	0	3 (100)	0
	MLV	4	0	4 (100)	0	4 (100)	0	4 (100)

^a A couple consisted of a patient and a sexual partner. NG-MLVA, *N. gonorrhoeae* multiple-locus variable-number tandem-repeat analysis; NLV, no locus variant; SLV, single-locus variant; DLV, double-locus variant; MLV, multilocus variant; NG-MAST, *N. gonorrhoeae* multiantigen sequence typing.

^b Number of patients or couples that had sets of isolates that were considered identical (NLV + SLV) or had DLVs or MLVs by NG-MLVA typing.

^c Numbers and percentages of patients or couples with different NG-MLVA types that were identical or nonidentical using NG-MAST and *porB* sequencing.

MAST STs, and *porB* sequences of the pre- and posttreatment isolates were identical for each case, with the exception of one case of cefixime treatment failure (20 days between sampling) in which an SLV in VNTR07-02 was found.

The 2008 WHO *N. gonorrhoeae* reference strains were included as quality controls and assigned eight different NG-MAST STs and *porB* sequences as previously described (22). In the present study, these were also distinguished as eight different NG-MLVA genotypes.

The discriminatory power of each typing method was determined by calculating the Simpson's diversity index (DI) using all 341 clinical isolates examined (3, 19). The DIs of NG-MLVA, NG-MAST, and full-length *porB* sequencing were 0.995 (95% confidence interval [CI], 0.994 to 0.996), 0.981 (95% CI, 0.976 to 0.986), and 0.979 (95% CI, 0.974 to 0.983), respectively. However, as previously described, the NG-MLVA types were epidemiologically best interpreted using hierarchical clustering, in which NG-MLVA types with an SLV were considered indistinguishable *N. gonorrhoeae* strains. For hierarchical clustering, a double weight was assigned to the more stable loci VNTR15-02 and VNTR16-01 (8). Using this interpretation of the NG-MLVA, the DI was 0.973 (95% CI, 0.966 to 0.980), i.e., in the same range as those from NG-MAST and *porB* sequence typing. Finally, in the entire sample material examined, we found 97.6% of the identified SLVs and double-locus variants (DLVs) to be due to polymorphisms in the number of short sequence repeats, i.e., the loci VNTR04-03, VNTR04-10, and/or VNTR07-02. NG-MAST identified 98 different STs, of which 59 (60.2%) were not previously described (www.ng-mast.net).

The selection of an appropriate typing method strongly depends on the epidemiological questions to be answered, but a single genotyping method will presumably never be able to, at the same time, answer all questions pertaining to both micro- and macroepidemiology (21). To monitor *N. gonorrhoeae* transmission in both low- and high-frequency transmitting populations, highly polymorphic loci examined by NG-MAST, *porB* sequencing, and NG-MLVA provide a high and sufficient discriminatory power (8, 13, 15, 21). However, too-rapid evolution of these loci might obscure *N. gonorrhoeae* transmission patterns within the total framework of the population under scrutiny (14, 21). We demonstrated this *in vitro* by performing long-term culture experiments and noted that the three highly variable VNTR regions showed polymorphism after ≥ 1 month (data not shown). This may reflect what could happen within one patient or

between couples. That is why we also in this study allowed one SLV to differ between isolates that were considered to belong to an indistinguishable strain, i.e., interpreted as an identical NG-MLVA type (8). In this way, the concordances of NG-MLVA with NG-MAST for patients with *N. gonorrhoeae* infection at multiple anatomical locations (118 patients) and for the 54 couples (108 isolates) were 83.1% and 83.3%, respectively, which is satisfactorily high. Furthermore, the concordances of NG-MLVA with *porB* sequencing were also high, i.e., 81.0% and 88.9%, respectively, for anatomical locations and couples. Comparing NG-MAST and *porB* sequencing, the concordances were 89.0% for multiple anatomical locations and 87.0% for couples. Overall, for 90/118 (76.3%) patients with infection at multiple anatomical locations and 43/54 (79.6%) couples, fully concordant cluster classifications were obtained by NG-MLVA, NG-MAST, and *porB* gene sequencing. The results thus showed that all three typing techniques have sufficiently high discriminatory power to differentiate distinct clusters of *N. gonorrhoeae* strains and provide similar cluster patterns for the large majority of isolates. Yet, some of the identical NG-MLVA types were further differentiated by NG-MAST and *porB* gene sequencing due mainly to single nucleotide polymorphisms (SNPs) in *porB*. However, this slightly elevated level of discrimination may only be needed in rare cases of extreme microepidemiology.

Interestingly, in one of three transmission chains that were typed to study locus stability, two isolates that were sampled 9 months apart were identified as indistinguishable by using both NG-MAST and *porB* gene sequencing, whereas NG-MLVA identified an MLV. These results show that four out of five NG-MLVA VNTR loci slightly evolved, while *porB* and *tbpB* remained stable. The isolates in one transmission chain (four months apart) and the pre- and posttreatment isolates of one case with cefixime treatment failure were identified as indistinguishable using both NG-MAST and *porB* gene sequencing, whereas NG-MLVA identified an SLV in the highly polymorphic VNTR07-02, which has previously been shown to evolve rapidly (8). This also confirms the need for accepting an SLV in NG-MLVA typing for identity. Furthermore, we observed that the NG-MLVA types of 6% of the patients with infections at different anatomical sites and 6% of the couples contained a DLV or MLV, whereas NG-MAST STs and *porB* sequences were identical. These locus variants might obscure transmission links and cause an underestimation of the real cluster size. Combining some of the markers of NG-MLVA, NG-MAST, and/or *porB* gene sequencing might resolve some of this instability and could prove to be valuable in the future

assessment of gonorrhea epidemiology. Furthermore, additional full *N. gonorrhoeae* genome sequences will become available soon, creating the opportunity to identify other VNTR markers that might further increase the power and robustness of the NG-MLVA (2).

Although NG-MAST, *porB* gene sequencing, and NG-MLVA are all considered relatively portable and cost-effective compared to many other *N. gonorrhoeae* typing techniques, important technical differences do exist. For NG-MAST, two PCRs and four sequencing reactions should be performed, whereas *porB* gene sequencing involves a single PCR and two to four sequence reactions, depending on the length of *porB* sequence examined. Sequence-based genotyping is considered the most precise and objective method for studying molecular epidemiology, including diversification and evolution of strains (21). However, NG-MLVA involves only fragment analysis following two multiplex PCRs. This provides fast and cost-effective genotyping, making NG-MLVA a very suitable candidate for high-throughput typing.

In conclusion, NG-MLVA, NG-MAST, and full-length *porB* sequencing are discriminatory and portable genotyping techniques that have excellent typeability. They can be used either as single typing methods or in combination to enhance the discriminatory ability, depending on the clinical, epidemiological, or scientific questions.

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