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Molecular characterization of *Neisseria gonorrhoeae* identifies transmission and resistance of one ciprofloxacin-resistant strain

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Abstract

A highly discriminative and objective genetic characterization of *N. gonorrhoeae*, which increases our knowledge of strain populations in different geographic areas, is crucial for the development of improved control measures. In the present study, conventional phenotypic characterization and genetic characterization by means of pulsed-field gel electrophoresis (PFGE), sequencing of the entire *porB* gene, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), and pyrosequencing of a quinolone resistance determining region (QRDR) of the *gyrA* gene of Swedish ciprofloxacin-resistant *N. gonorrhoeae* serovar IB-10 isolates (n=45) were performed. The genetic characterization identified one widely spread ciprofloxacin-resistant *N. gonorrhoeae* ST147 strain. In addition, isolates with slightly different genetic characteristics, which presumably reflect the ongoing evolution only, were also identified. All the isolates contained single nucleotide polymorphisms in QRDR of the *gyrA* gene that are highly correlated with ciprofloxacin resistance. Consequently, comprehensive characterization identified the first confirmed large domestic transmission, mainly among young heterosexuals, of one ciprofloxacin-resistant *N. gonorrhoeae* strain in Swedish society during 2002–2003. In conclusion, a precise, i.e. genetic, characterization for identification of individual strains is a very valuable support to the crucial active surveillance of the epidemiological characteristics and the antibiotic susceptibility of *N. gonorrhoeae* in the effective treatment of gonorrhoea.

Keywords

Neisseria gonorrhoeae; ciprofloxacin resistance; molecular epidemiology; *porB* gene; NG-MAST

Gonorrhoea remains a major sexually transmitted infection (STI) in many countries (1,2). The mainstay in its prevention is based on the availability of effective diagnostics and antibiotic treatment, as well as regional, national, and international surveillance of the epidemiological characteristics and the antibiotic susceptibility of the etiological agent, i.e. *Neisseria gonorrhoeae*. Nowadays, a high level of decreased susceptibility or resistance to most traditional antibiotics used for treatment of gonorrhoea has been identified in most countries

(SWEDRES 2004 <http://www.strama.se> (2–5)). In addition, the characteristics of circulating *N. gonorrhoeae* strains fluctuate over time and a highly discriminative and objective characterization, i.e. genetic typing, of the bacteria, is crucial. This genetic typing may identify the transmission of more virulent and/or antibiotic-resistant strains, information that is important for the development of improved control measures. Conventional phenotypic characterization of *N. gonorrhoeae* strains has several limitations (6–10) and many genetic methods have been developed to overcome these shortcomings (11).

Since 1997 the incidence of gonorrhoea in Sweden has increased almost annually (Gonorrhoea/year and month statistics per county http://gis.smittskyddsinstitutet.se/mapapp/build/intro_eng.html (3,8)). According to previous studies (8,10,12), the transmission of single strains in Swedish society may result in large domestic core groups of gonorrhoea cases and it is possible to identify these strains with a precise genetic characterization of the bacterial isolates. Since the mid-1990s, the prevalence of isolates resistant to ciprofloxacin, previously the drug of first choice for treatment of uncomplicated gonorrhoea, has been increasing in Sweden and in many other countries (SWEDRES 2004 <http://www.strama.se> (2–5,13)). In *N. gonorrhoeae*, the ciprofloxacin resistance is mainly due to single nucleotide polymorphisms (SNPs) in the *gyrA* gene and the *parC* gene, which code for the target enzymes DNA gyrase and topoisomerase IV, respectively (14–19). The level of resistance appears to be correlated with the location and number of mutations in these genes. However, treatment failures with fluoroquinolones have been shown to be due to mutations primarily in the *gyrA* gene, and especially substitutions in a short quinolone resistance determining region (QRDR) of this gene (14,19,20). Consequently, it may be sufficient to analyze a short QRDR of the *gyrA* gene for identification of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to ciprofloxacin (14–19,21,22).

In 2003, a presumed epidemiological core group comprising domestic cases of mainly young (<25 years of age) heterosexuals with urogenital gonorrhoea was identified in one Swedish county. All preserved *N. gonorrhoeae* isolates from these cases were assigned to serovar IB-10 and showed a high level of resistance to ciprofloxacin. During 2002 and 2003, many IB-10 isolates that showed a high level of resistance to ciprofloxacin were also identified in other geographic areas of Sweden.

The aim of the present study was to thoroughly characterize all ciprofloxacin-resistant *N. gonorrhoeae* IB-10 isolates from an epidemiologically identified presumed core group in a Swedish county in 2003 as well as from other geographic areas of Sweden during 2002 and 2003. This was done in order to investigate the possibility of wide transmission, mainly among young (<25 years of age) heterosexuals, of one ciprofloxacin-resistant *N. gonorrhoeae* strain in Swedish society.

Materials and Methods

Bacterial isolates and culture conditions

In the present study, clinical ciprofloxacin-resistant *N. gonorrhoeae* IB-10 isolates (n=45) and clinical ciprofloxacin-susceptible *N. gonorrhoeae* IB-10 isolates (n=2; 165/03 and 177/03) were examined (Fig. 1). The isolates included all ciprofloxacin-resistant *N. gonorrhoeae* IB-10 isolates cultured and preserved in Sweden during 2002 and 2003. These were initially isolated on selective culture medium and species verified according to standard procedures at many different clinical microbiology laboratories in Sweden. Subsequently, at the Swedish Reference Laboratory for Pathogenic Neisseria, Department of Clinical Microbiology, Örebro University Hospital, all isolates were species confirmed using sugar utilisation test and Phadebact GC Monoclonal Test (Boule Diagnostics AB, Huddinge, Sweden). 19 of the isolates were collected from an epidemiologically identified presumed core group of domestic

gonorrhoea cases (n=19) in Gävleborg county, 170 km north of the Swedish capital city Stockholm, during late winter and early spring 2003. One *N. gonorrhoeae* IB-10 isolate was cultured from an additional patient presumed to belong to the suspected core group; however, in the present study this patient was excluded because the isolate was not available. The suspected core group comprised mainly young heterosexuals (6 males and 12 females; 16/18 [89%] <25 years of age) with urogenital gonorrhoea but also a neonatal boy suffering from conjunctivitis. The remaining ciprofloxacin-resistant isolates (n=26) were cultured from gonorrhoea patients (19 males and 7 females; 16/26 [62%] <25 years of age) in 10 other cities located in both the southern and northern part of Sweden during 2002 and 2003. In addition, two clinical ciprofloxacin-susceptible (165/03 and 177/03; MIC=0.004 mg/l) *N. gonorrhoeae* IB-10 isolates that circulated in the Swedish society in 2003 were included for comparison. All isolates were preserved at -70°C and cultured as previously described (10).

Epidemiological information and demographic data

Gonorrhoea is a mandatorily notifiable disease according to the Swedish Communicable Diseases Act. In the present study, epidemiological and demographic data were collected for all gonorrhoea cases from case reports at the Department of Epidemiology, the Swedish Institute for Infectious Disease Control. In addition, for the presumed core group patient information regarding partner tracing was collected from the counsellors handling each individual case.

Phenotypic antibiotic susceptibility testing

β -lactamase production was analyzed with Nitrocefin discs (AB Biodisk, Solna, Sweden) and susceptibility to ciprofloxacin, ampicillin, cefixime, ceftriaxone, azithromycin, and spectinomycin was analyzed using the Etest method (AB Biodisk), as previously described (3).

Serological characterization

Serovar determination was performed using the co-agglutination technique (23) with the Pharmacia panel (Ph) (24) (Bactus AB, Stockholm, Sweden) and the Genetic Systems (GS) panel (25) of monoclonal antibodies (MAbs).

Pulsed-field gel electrophoresis (PFGE)

PFGE of the *N. gonorrhoeae* isolates, with the two restriction endonucleases *Spe*I and *Bgl*III used separately, was performed, documented, and analyzed as previously described (8). Isolates were considered genetically indistinguishable if no band differed in any of the fingerprints (identical PFGE type, e.g. A), closely related if 1–3 bands differed in any or both of the fingerprints (variant of the PFGE type, i.e. A1, A2, A3), possibly related if 4–6 bands differed in any or both of the fingerprints (variant of the PFGE type, i.e. A4, A5, A6, A7, A8) and different if ≥ 7 bands difference was documented in any of the fingerprints, e.g. PFGE type B (26) (Fig. 1).

Isolation of genomic DNA

Bacterial DNA was isolated using magnetic silica particles in a robotized system (MagNA Pure LC, Roche Molecular Biochemicals, Mannheim, Germany), as previously described (21). The DNA preparations were stored at 4 °C prior to PCR.

Real-time PCR of the entire *porB* gene

The entire *porB* gene that codes for the outer membrane porin PorB was amplified in a LightCycler System (Roche Molecular Biochemicals) using SYBR Green I fluorescence

melting curve analysis for specific identification of the *porB1b* amplicons, with the primers PorBU and PorBL as previously described (27). The PCR products were stored at 4 °C prior to purification.

***N. gonorrhoeae* multiantigen sequence typing (NG-MAST)**

The more variable internal segment of the *porB* gene and the *tbpB* gene that code for the β subunit of the transferring-binding protein, respectively, which are analyzed in the NG-MAST protocol (28), was PCR amplified in a LightCycler System (Roche Molecular Biochemicals) using SYBR Green I fluorescence melting curve analysis for specific identification of the amplicons. In the amplifications, the previously documented primers (28) *por* forward and *por* reverse and *tbpB* forward and *tbpB* reverse, respectively, were used. Briefly, each PCR mixture (20 μ l) contained: 2 μ l LightCycler – FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH), 3 mM MgCl₂ (Roche Diagnostics GmbH), 0.5 μ M of each primer, and 2 μ l of DNA template. The cycling parameters of the amplifications were as follows: an enzyme activation step at 95 °C for 10 min, followed by 30 sequential cycles of heating up to 95 °C, 58 °C (69 °C for the *tbpB* gene) for 10 s, and 72 °C for 30 s (24 s for the *tbpB* gene). The cycling parameters of the subsequent melting curve analysis were as follows: heating the PCR products up to 95 °C, cooling at 63 °C (74 °C for the *tbpB* gene) for 45 s, and finally slowly heating (0.1 °C/s) up to 95 °C.

A positive control (DNA of *N. gonorrhoeae* reference strain CCUG 15821) and a negative control (distilled water) were included in each PCR run. The PCR products were stored at 4 °C prior to purification.

***gyrA* PCR**

The QRDR of the *gyrA* gene was amplified using the primers GYRA2-1 and GYRA2-2 (biotin-labeled for single-strand separation) as previously described (21). The PCR products were stored at 4 °C prior to sequencing using Pyrosequencing technology.

Conventional Sanger-dideoxy sequencing

The amplified *porB1b* allele was cycle sequenced using the primers PorBU, PorBL, PorB1bU, and PorB1bL (10) and the more variable segments of the *porB* gene and the *tbpB* gene, analyzed in the NG-MAST protocol (28), were cycle sequenced with the same primers as used in the PCR as previously described (29). The purification of the PCR products and the sequence extension products as well as the sequence determination with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) were performed as previously documented (29). The sequence of each strand of each compiled sequence was determined.

***gyrA* sequencing by Pyrosequencing technology**

The Pyrosequencing method is a real-time DNA sequencing-by-synthesis technology (21,27,30). As previously described (21), single-strand DNA sample preparation prior to sequencing was performed semi-automatically on a Vacuum Prep Workstation (Biotage, Uppsala, Sweden) using streptavidin sepharose beads (Amersham Biosciences, Uppsala, Sweden). The single-strand prepared PCR products from the isolates were sequenced, with the primer GYRA2–3, using the PSQ 96 SNP reagent kit (Biotage) and an automated plate-based bench-top PSQ™ HS96A System (Biotage), as previously documented (21). Result analysis was performed automatically with SNP PSQ™ HS96A 1.2 software and by visual inspection. The wild-type *gyrA* gene in the ciprofloxacin-susceptible *N. gonorrhoeae* reference strain FA 1090 (ATCC 700825) was used for evaluation of the results.

Sequence analysis and phylogenetic inference

Multiple-sequence alignments of the entire *porB* gene segments encoding the mature PorB (10), the more variable regions of the *porB* gene and the *thpB* gene analyzed in the NG-MAST protocol (28), and the sequences of the QRDR in the *gyrA* gene were performed with BioEdit (version 5.0.9) software and by manual adjustment. Construction of the phylogenetic tree of the entire *porB* gene sequences was performed with TREECON (version 1.3b) software (31), as previously described (9). The *porB* gene sequence of an *N. gonorrhoeae* serovar IB-9 reference strain (CCUG 15821) was used as an outgroup to root the tree.

The more variable segments of the *porB* gene (490 bp) and the *thpB* gene (390 bp), analyzed in the NG-MAST protocol (28), in all isolates were compared to identify polymorphisms. By using the NG-MAST website (available at <http://www.ng-mast.net>), each isolate was assigned to a sequence type (ST), which is defined by the allelic profile present at the two different loci.

Evaluation of data

All data regarding patients and isolate characteristics were compiled and evaluated in an Excel database.

Results

Characterization of ciprofloxacin-resistant *N. gonorrhoeae* IB-10 isolates from gonorrhoea cases from a suspected domestic core group in a Swedish county in 2003 (n=19)

The IB-10 isolates (n=19) were all assigned to serovar Bropst with Ph MAbs (Fig. 1). In addition, all isolates were β -lactamase negative, highly resistant to ciprofloxacin ($\text{MIC} \geq 4$ mg/l), showed decreased susceptibility to ampicillin ($\text{MIC} \leq 0.125$ mg/l), and full susceptibility to cefixime ($\text{MIC} \leq 0.064$ mg/l), ceftriaxone ($\text{MIC} \leq 0.064$ mg/l), azithromycin ($\text{MIC} \leq 0.5$ mg/l), and spectinomycin ($\text{MIC} \leq 32$ mg/l). Furthermore, all isolates showed similar MIC values, with differences within ± 1 log₂.

According to the genetic characterization, all the isolates comprised identical *porB* gene sequences and were assigned to ST147 in the NG-MAST (Fig. 1). The 19 isolates comprised two distinguishable PFGE fingerprints with use of *Bgl*II and three with *Spe*I. However, 18 (95%) of the isolates showed indistinguishable fingerprints with use of *Bgl*II and 13 (68%) with *Spe*I. In addition, all fingerprints differed by a maximum of two bands. Consequently, 13 (68%) of the isolates were indistinguishable (PFGE-type A) and the remaining six isolates were closely related, i.e. interpreted as two variants of the same PFGE-type (A1 and A2), when the results of both enzymes were combined (Fig. 1).

Characterization of ciprofloxacin-resistant *N. gonorrhoeae* IB-10 isolates from other geographic areas of Sweden in 2002-2003 (n=26)

24 (92%) of the IB-10 isolates (n=26) were assigned to Ph serovar Bropst and two were assigned to Ph serovar Bropt, i.e. differed by the reactivity of one MAb only (Fig. 1). Nine (35%) of the isolates were β -lactamase negative, highly resistant to ciprofloxacin ($\text{MIC} \geq 1.5$ mg/l), showed decreased susceptibility to ampicillin, and full susceptibility to cefixime, ceftriaxone, azithromycin, and spectinomycin. The remaining 17 isolates (65%) produced β -lactamase. However, except for this difference, all of the isolates showed MICs similar to those of the β -lactamase-negative core group isolates to the other antibiotics.

According to the genetic characterization, the isolates (n=26) possessed four slightly different *porB* gene sequences (differed by one to two nucleotides) and were assigned to four different STs (ST147, ST341, ST674, and ST900; Fig. 1). However, 22 (85%) of the isolates revealed a *porB* gene sequence identical to that of the core group isolates mentioned above (Fig. 1). In

addition, 21 (81%) of the isolates were assigned to ST147, i.e. the same ST as the core group isolates, and three of the remaining five isolates only differed by a maximum of one nucleotide in the *porB* segment and comprised identical sequences in the *tbpB* segment, i.e. had closely related STs (ST674 and ST900; Fig. 1). However, the last two isolates, i.e. 167/02 and 124/03, that were assigned to ST341 had an identical *porB* gene sequence but the *tbpB* segment differed by 77 nucleotides (Fig. 1). The 26 isolates possessed six distinguishable PFGE patterns with use of *Bgl*III and three with *Spe*I, but 18 (69%) of the isolates were indistinguishable from any of the core group isolates. In addition, the fingerprints of all the 26 isolates differed by less than 7 bands (both enzymes used) from the fingerprints of the core group isolates, i.e. were possibly related. Consequently, these fingerprints were interpreted as PFGE variants of the same type (Fig. 1).

The two Swedish ciprofloxacin-susceptible IB-10 isolates from 2003 (165/03 and 177/03; Fig. 1), which were included for comparison, had similar antibiotic susceptibility patterns, with the exception of ciprofloxacin, to those of the ciprofloxacin-resistant IB-10 isolates. However, these isolates differed substantially in the entire *porB* gene, were assigned to the highly divergent ST259, and the PFGE fingerprints differed significantly from those of the core group isolates, i.e. by more than seven bands with both enzymes (Fig. 1).

Pyrosequencing of QRDR of the *gyrA* gene in *N. gonorrhoeae* IB-10 isolates (n=47)

All the phenotypically determined ciprofloxacin-resistant *N. gonorrhoeae* IB-10 isolates (n=45) contained two nonsynonymous SNPs in the examined QRDR of the *gyrA* gene, i.e. in the Ser91 and the Asp95 codons of the wild-type *gyrA* gene sequence (Table 1). However, two different polymorphic variants of the Asp95 codon were identified. Consequently, 43 of the isolates contained Gly95 while the two remaining isolates contained Ala95 (Table 1). The two ciprofloxacin-susceptible IB-10 isolates, included for comparison, comprised the wild-type QRDR of the *gyrA* gene (Table 1).

Discussion

In the present study, thorough genetic characterization together with phenotypic and epidemiological information identified a transmission of one ciprofloxacin-resistant *N. gonorrhoeae* serovar IB-10 ST147 strain in Swedish society during 2002 and 2003. This was the first confirmed large domestic spread of one ciprofloxacin-resistant *N. gonorrhoeae* strain in Sweden. In 2003, transmission of the strain caused the gonorrhoea cases in a domestic core group comprising mainly young (<25 years of age) heterosexuals in one Swedish county. The majority of these patients showed epidemiological connections with each other according to the partner notification investigations. The strain was also identified in several other Swedish counties during 2002 and 2003, in a total of 10 different cities located in both the southern and northern part of Sweden. Minor phenotypic and genetic diversities of some isolates of the strain were identified. For instance, some isolates seemed to have acquired a β -lactamase plasmid and detailed genetic analysis revealed some minor polymorphisms in the *porB1b* gene, the *tbpB* gene, the QRDR of the *gyrA* gene, and the PFGE fingerprints. This limited variation probably reflects ongoing evolution of the same strain only, which also the phylogenetic analysis of the *porB1b* gene sequences suggested. Most interesting, many isolates of ciprofloxacin-resistant *N. gonorrhoeae* ST147 were also identified in 2000–2004 in London, England (32,33) and in 2002 in Scotland (34), which may indicate import of this strain to Sweden. In London, England, the transmission of the ciprofloxacin-resistant ST147 isolates was predominantly by homosexual men (32), whereas in Scotland mainly heterosexual transmission occurred (34). However, none of the Swedish patients reported a sexual contact in England or Scotland.

Overall, the results of the sequencing of the entire *porB* gene, NG-MAST, and PFGE showed congruence but not complete identity, which is in concordance with previous studies (10,35–37). All these genetic methods exhibited a high discriminatory ability and excellent typeability, which also agree with previous studies (8–10,28,34–38). However, when characterizing *N. gonorrhoeae* for epidemiological purposes, a sequence-based genetic typing seems to be the best single choice of method. This is due to the fact that sequence data are also objective, highly reproducible, and portable for comparison between laboratories. In comparison with *porB* gene sequencing, NG-MAST has the advantage of analyzing segments of two different genes. Consequently, NG-MAST can identify that highly divergent sequences in one of the genetic loci may be due to a single genetic event, i.e. by genetic exchange and/or recombination, and, if there are identical sequences in the other locus, isolates may in fact still be closely related. Gel-based genetic typing such as PFGE and *opa* typing (39) is laborious, time-consuming, subjectively interpreted, and requires pronounced standardization, especially for interlaboratory comparisons. However, PFGE and *opa* typing may reveal a somewhat higher discrimination between isolates compared to sequencing of the entire *porB* gene and NG-MAST (present study, 28,36). Consequently, in some situations, especially involving short-term epidemiology, these methods may provide a useful complement to sequence-based approaches for characterization. In the present study, the slightly divergent PFGE fingerprints of some of the isolates probably reflect that point mutations or recombination have occurred causing a distinct evolutionary history in the *porB1b* gene and/or the *tbpB* gene in comparison to other parts of the genome. No restriction sites for the PFGE enzymes *SpeI* and *BglII* existed in the *porB1b* genes or the *tbpB* genes of the examined isolates. Thus, the results of the present study as well as numerous previous studies (8–10,12,28,34–38) emphasize that a highly discriminative, reproducible, and objective characterization of *N. gonorrhoeae* is crucial for understanding gonorrhoea epidemiology. A precise method, i.e. genetic, for short-term epidemiological characterization of *N. gonorrhoeae* is a necessary tool for conducting efficient contact tracing, identification of core groups or clusters of gonorrhoea cases, performance of precise epidemiological surveillance, and identification of the spread of, for example, more virulent and/or antibiotic-resistant strains. This information is important for development of improved control measures.

In the present study, the QRDR of the *gyrA* gene in all isolates was successfully sequenced using Pyrosequencing technology. All phenotypically determined ciprofloxacin-resistant isolates comprised SNPs that are highly correlated with ciprofloxacin resistance, i.e. in the Ser91 and the Asp95 codons of the wild-type *gyrA* gene sequence (14–19,21,22). This method based on Pyrosequencing technology is rapid, high-throughput, sensitive, accurate, and cost-effective for identification of ciprofloxacin resistance in *N. gonorrhoeae*. However, a few *N. gonorrhoeae* isolates with decreased susceptibility or resistance to ciprofloxacin that lack substitutions in the QRDR of the *gyrA* gene as well as in the *parC* gene have previously been described (40–42). These very rare isolates result in discrepancies between phenotypic and genetic ciprofloxacin susceptibility testing and may be due to polymorphisms in other regions of the *gyrA* gene or *parC* gene, or polymorphisms in other genes such as the *porB* gene (20, 40,43–45). Whilst culture remains the gold standard for diagnosis of *N. gonorrhoeae*, an increasing number of laboratories are using molecular methods for diagnosis. Consequently, effective molecular methods for determination of the antibiotic susceptibility of *N. gonorrhoeae* and for precise epidemiological characterization of *N. gonorrhoeae* from clinical samples need to be developed.

With the exception of ciprofloxacin, the disseminated strain identified in the present study did not show any resistance to antibiotics currently used for treatment of gonorrhoea. In Sweden, mainly ceftriaxone, cefotaxime, spectinomycin, cefixime, azithromycin—or in some cases still ciprofloxacin—are used for treatment of gonorrhoea. Previously, ciprofloxacin was the drug of first choice, but resistance to this antibiotic has rapidly increased (SWEDRES 2004

<http://www.strama.se> (3,12)). In addition, domestic transmission of azithromycin-resistant *N. gonorrhoeae* strains has recently been described (12). Consequently, at present ceftriaxone, spectinomycin, or cefixime can be recommended as the drug of first choice for treatment of gonorrhoea in Sweden in situations when the result of antibiotic susceptibility testing is still pending.

In conclusion, a thorough genetic characterization together with phenotypic and epidemiological information identified the first confirmed large domestic transmission of a ciprofloxacin-resistant *N. gonorrhoeae* strain in Sweden during 2002 and 2003. Whilst minor phenotypic and genetic diversities of some isolates of the strain were identified, this presumably only reflects the ongoing evolution. The present study emphasizes that a precise, i.e. genetic, characterization of *N. gonorrhoeae* for identification of individual strains is a very valuable support to the crucial active surveillance of the epidemiological characteristics and the antibiotic susceptibility of the bacteria.

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Fig. 1.

Neighbor-joining phylogenetic tree describing the evolutionary relationship of *porB1b* gene sequences (993 unambiguously aligned nucleotides) coding for the mature PorB (10) of clinical *Neisseria gonorrhoeae* serovar IB-10 isolates (n=47). The *porB1b* sequence of the *N. gonorrhoeae* IB-9 reference strain (CCUG 15821), which represents an outgroup, was used to root the tree. The length of the branches leading to the outgroup has been reduced by a factor of four. The *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) sequence types (STs) are denoted. The pulsed-field gel electrophoresis (PFGE) types (no band difference, e.g. A) and variants (e.g. A1), i.e. less than seven bands difference in both the *SpeI* and the *BglII* fingerprints, are shown. The serovars of the isolates using Pharmacia (Ph) MAbs are indicated

in italics. The designation of each isolate (laboratory number/year of isolation) and the susceptibility to ciprofloxacin (CipS, susceptible; CipR, resistant) are also shown. The isolates that originated in the domestic core group in a Swedish county in 2003 are shaded. *, indicates not previously identified ST.

DNA sequences encoding amino acid position 90–96 in the quinolone resistance determining region (QRDR) of the *gyrA* gene in *N. gonorrhoeae* IB-10 isolates (n=47)

	Amino acid position in GyrA						
	90	91	92	93	94	95	96
Ciprofloxacin-susceptible isolates (n=2) ^d	GAT	TCC	GCA	GTT	TAC	GAC	ACC
Ciprofloxacin-resistant isolates (n=45) ^b	(Asp)	(Ser)	(Ala)	(Val)	(Tyr)	(Asp)	(Thr)
Variant 1 (n=43)	GAT	TTC	GCA	GTT	TAC	GGC	ACC
	(Asp)	(Phe)	(Ala)	(Val)	(Tyr)	(Gly)	(Thr)
Variant 2 (n=2) ^c	GAT	TTC	GCA	GTT	TAC	GCC	ACC
	(Asp)	(Phe)	(Ala)	(Val)	(Tyr)	(Ala)	(Thr)

^aThe ciprofloxacin-susceptible isolates had MICs of 0.004 and comprised a wild-type *gyrA* gene segment, i.e. identical to the one in the ciprofloxacin-susceptible *N. gonorrhoeae* reference strain FA 1090 (ATCC 700825).

^bThe ciprofloxacin-resistant isolates showed MICs of 1.5–16. All of these isolates comprised nonsynonymous nucleotide substitutions that are highly correlated with ciprofloxacin resistance in *N. gonorrhoeae*, i.e. in amino acid codon 91 and 95. These substitutions are indicated by underlined, boldface capital letters.

^cVariant 2 was identified in one of the isolates originating in the Swedish core group (163/03; see Fig. 1) and in one isolate from 2002 (179/02; see Fig. 1). The encoded amino acids are shown in parentheses.