Clonally related *Neisseria gonorrhoeae* isolates with decreased susceptibility to the extended-spectrum cephalosporin cefotaxime in Amsterdam, the Netherlands

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The present study was performed at the Public Health Laboratory, Health Service of Amsterdam, the Netherlands and the WHO Collaborating Centre for Gonorrhoea and other STIs, Örebro University Hospital, Örebro, Sweden.

Running title: Spread of gonococcal *penA* mosaic strain in Amsterdam

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From 2006 to 2008, *Neisseria gonorrhoeae* (NG) isolates were identified with decreased susceptibility to the extended-spectrum cephalosporin (ESC) ceftaxime among visitors of the Amsterdam STI clinic, the Netherlands. Spread, clonality, and characteristics of 202 isolates were examined using antibiograms, conventional penA mosaic gene PCR, and NG multiple-locus variable-number tandem repeat analysis (NG-MLVA). A strictly defined subset was further characterized by NG multilocus sequence typing (NG-MAST) and sequencing of ESC resistance determinants (penA, mtrR, and porB1b). Seventy-four NG isolates with a ceftaxime MIC of >0.125 µg/ml (group A), 54 with a ceftaxime MIC of 0.125 µg/ml (group B), and a control group of 74 with a ceftaxime MIC of <0.125 µg/ml (group C), were included. Fifty-three clonally related penA mosaic positive isolates (penicillin-binding protein 2 type XXXIV) were identified in group A (n=47; 64%) and B (n=6; 11%). The 53 penA mosaic positive isolates were predominantly NG-MAST ST1407 (87%) and contained an mtrR promoter A-deletion (98%) and porB1b alterations G101K/A102N. All were assigned to the same NG-MLVA cluster that comprised in total 56 isolates. A correlation was found between decreased ceftaxime susceptibility and ST1407 that was highly prevalent among visitors of the Amsterdam STI clinic. The rapid spread of this strain, which also has been identified in many other countries, might be facilitated by high-risk sexual behaviour and should be monitored closely to identify potential treatment failure. Quality-assured surveillance of ESC susceptibility on a national and international level and exploration of new drugs and/or strategies for treatment of gonorrhea are crucial.
Neisseria gonorrhoeae is the etiological agent of the sexually transmitted infection (STI) gonorrhea. During the last decades, N. gonorrhoeae has effectively developed plasmid-mediated and/or chromosomally mediated antibiotic resistance to penicillins, tetracyclines, fluoroquinolones, and most other antibiotics used for treatment of gonorrhea (3,16,25). This loss of treatment efficacy led in many countries to discontinuation of these antibiotics as standard first-line treatment regimens, posing new public health concerns (5,25,35).

At present, in most countries extended-spectrum cephalosporins (ESCs), such as cefixime (oral) and ceftriaxone (injectable), are the first-line treatment for gonorrhea (3,4,25). In the Netherlands, until 2006 the national clinical guidelines recommended cefotaxime as first-line therapy for gonorrhea. However, since 2006 ceftriaxone is available as an intramuscular injection in the Netherlands and is recommended as first-line treatment (8). Although parenteral ESCs, such as ceftriaxone and cefotaxime, generally show good clinical efficacy for gonococcal infection, various studies have reported the emergence of N. gonorrhoeae strains with decreased susceptibility to these antibiotics (10,17,20,21,24,25,33,38). Worryingly, a recent report described the first European case (in Sweden) of verified treatment failure of pharyngeal gonorrhea using ceftriaxone (29), and the first strain worldwide with high-level resistance to ceftriaxone has recently been reported from Japan (20,29).

One important antibiotic resistance determinant that has been associated with this decreased susceptibility and resistance to ESCs in gonococci is the penA mosaic gene, which encodes a mosaic variant of penicillin binding protein 2 (PBP2), the primary lethal target of β-lactam antibiotics (2,14). Polymorphisms in at least three important mosaic PBP2 residues (I312M, V316T, and G545S) and epistasis of the polymorphisms in mosaic PBP2 variants cause a marked decrease in susceptibility to cefixime and ceftriaxone (23,27). However, additional polymorphisms in the penA gene, e.g. alterations in A501 in PBP2, are important,
and still a lot of knowledge is lacking. Yet, with exception of the Japanese strain (19) it has been well established that the identified alterations in the penA mosaic gene do not seem to be sufficient to attain very high level of ESC resistance without the contribution of mtrR, porB1b, and at least one additional unknown resistance determinant (10,17,20,25,38).

Since 2006, cefotaxime is used to monitor susceptibility to ESCs in the Netherlands. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI), US have defined different MIC resistance breakpoints for cefotaxime of >0.125 µg/ml and >0.5 µg/ml, respectively. Although N. gonorrhoeae isolates with a MIC over the CLSI breakpoint have not yet been found in the Netherlands, isolates with a MIC between 0.125 and 0.5 µg/ml have been frequently identified (6). In 2009, we reported a notable 7.3% increase in prevalence over a two-year period (2006 to 2008) of multidrug-resistant N. gonorrhoeae isolates with such a decreased susceptibility to cefotaxime among visitors of the STI outpatient clinic in Amsterdam (6). This sharp increase in prevalence was associated with high-risk sexual behaviour and STI co-infections, which suggested rapid clonal expansion of an antibiotic resistant N. gonorrhoeae strain, such as was described in other studies (10,13,14,36).

In the present study, we investigated the transmission patterns, clonality, and phenotypic as well as genotypic characteristics of N. gonorrhoeae isolates, which were classified as intermediate susceptible/resistant, borderline susceptible, and susceptible to cefotaxime, according to EUCAST criteria. All isolates were obtained from well defined high-risk populations in Amsterdam.
MATERIALS & METHODS

Study setting, population, and sampling. The STI outpatient clinic of the Amsterdam Public Health Service, the Netherlands, is a low-threshold clinic serving approximately 30,000 clients annually. During 2007 and 2008, a total of 3791 cases of gonorrhea were diagnosed in national public health settings, of which 2077 (55%) were diagnosed in the Amsterdam STI outpatient clinic. Clients can visit the STI clinic anonymously, free of charge, and without referral of a healthcare provider. Upon arrival, clients are prioritized based on a short questionnaire to estimate the risk for having an STI (11). All clients that are considered high-risk patients get a full STI check-up, including collection of swabs for *N. gonorrhoeae* cultivation and a detailed questionnaire concerning risk behavior. High-risk groups include all men who have sex with men (MSM), persons who are paid for having sex, persons with symptoms, persons who report that their partner has an STI, persons who have been referred to the outpatient department by another health care provider, and persons originating from Sub-Saharan Africa (increased risk for HIV, a rapid HIV test is offered). Only asymptomatic clients from low-risk groups are tested by NAAT only.

Patients diagnosed with a *N. gonorrhoeae* infection (urogenital, anorectal, and/or pharyngeal infection) are treated with 500 mg ceftriaxone intramuscularly according to the national guidelines of the Dutch Dermatological and Venereological Society (8). Azithromycin (1 g, single oral dose) as treatment for co-existing *Chlamydia trachomatis* infection is given to all patients with proven co-infection and as syndromic treatment in patients who are positive for gonorrhea in microscopy after Gram staining during the screening visit.

The samples and data for this study were collected as part of the routine clinical procedure; therefore no Ethical Committee approval was needed.
Bacterial cultivation, antimicrobial susceptibility, and lysate preparation. For the cultivation of *N. gonorrhoeae*, urethral, cervical, rectal, and/or pharyngeal swab specimens were directly inoculated onto GC-Lect agar plates (Becton Dickinson, USA) and incubated in an aerobic, carbon dioxide enriched environment at 37°C for 40-48 hours. The identification of *N. gonorrhoeae* was based on Gram-staining, oxidase-, sugar utilization-, and aminopeptidase reactions and a DNA probe test, in accordance to the instructions from the manufacturer (AccuProbe, Gen-Probe, USA). For all *N. gonorrhoeae* isolates, MICs of cefotaxime, ceftriaxone, cefixime, penicillin G, tetracycline, and ciprofloxacin were measured using the Etest method, in accordance to the instructions from the manufacturer (AB bioMérieux, Sweden). The 2008 WHO *N. gonorrhoeae* reference strains G, K, L, M, O and P (28) were used as control strains for all antimicrobial susceptibility testing. Since cefotaxime MICs were not available for these strains, we determined them for our conditions. WHO reference strains K and L had decreased susceptibility to extended-spectrum cephalosporins and had cefotaxime MICs of 0.19 and 0.25 µg/ml, respectively. The other strains had cefotaxime MICs varying from 0.006 to 0.047 µg/ml. Colonies of *N. gonorrhoeae* positive cultures were suspended in sterile saline (0.5 McFarland standard), lysed at 95°C for 10 min, and stored at -80°C prior to amplification.

Selected *N. gonorrhoeae* isolates from 2006 to 2008. To include the lower levels of decreased susceptibility and/or antimicrobial resistance, *N. gonorrhoeae* isolates were selected according to the EUCAST resistance breakpoint (v1.3) for cefotaxime (MIC >0.125 µg/ml), rather than using the higher resistance breakpoint issued by the CLSI (MIC >0.5 µg/ml). From October 2006 to December 2008, from 90 patients a *N. gonorrhoeae* isolate with an MIC of cefotaxime over 0.125 µg/ml and from 70 patients an isolate with an MIC of cefotaxime of 0.125 µg/ml had been cultured. Group A consisted of 74 (82%) isolates with an
MIC over 0.125 µg/ml which could be revived. Group B included 54 (77%) isolates with an 
MIC of 0.125 µg/ml which were still viable. Only one isolate per patient was included in the 
present study; if several isolates had been cultured from different locations of a single patient, 
the isolate with the highest MIC was chosen. Control group C consisted of 74 N. gonorrhoeae 
isolates with a cefotaxime MIC of <0.125 µg/ml. For each group A isolate, a control isolate 
was included within a three day time span from inclusion. Of the 202 isolates included in the 
present study, 96 (47.5%) were cultured from urethra, 87 (43.1%) from rectum, 10 (5.0%) 
from pharynx, and nine (4.5%) from cervix. The percentage of urethral isolates was 
significantly higher in group C (58%) in comparison to group A (41%, p=0.048). The number 
of rectal isolates was significantly higher in group A (51%) in comparison to group C (32%, 
p=0.030). Significantly more pharyngeal isolates were found in group B (9%) in comparison 
to group C (0%, p=0.012).

Detection of the penA mosaic gene. The detection of penA mosaic alleles in N. 
gonorrhoeae lysates was based on a protocol, previously described by Whiley et al. 2007 
(32). PCR was performed using a 25 µl reaction volume containing 2 µl of heat-treated N. 
gonorrhoeae lysates, 1 U Taq DNA polymerase (Promega, USA), 5 µl of 5x Flexi GoTaq 
buffer (Promega, USA), 2.5 mM MgCl2 solution (Promega, USA), 200 µM of dNTPs (Roche, 
Diagnostics, Switzerland), and 0.36 µM of forward primer penA-F, 0.04 µM each of reverse 
primers penA-R1 to R4, and 0.2 µM of reverse primer penA-R5. Amplification was 
performed on a Bio-Rad C1000 PCR System (Bio-Rad, USA) at 95°C for 2 min, followed by 
35 amplification cycles of 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C. The PCR products 
were electrophoresed on a 10% polyacrylamide gel (Bio-Rad, USA). WHO strains K and L 
were used as a positive and negative control, respectively. WHO K and L (penA D345a allele) 
strains both display decreased susceptibility to ESCs due to a penA mosaic allele and a penA 
A501V mutation, respectively (28).
N. gonorrhoeae MLVA. Amplification of the variable-number of tandem repeat sequences (VNTRs) was performed on a Bio-Rad C1000 PCR System (Bio-Rad, USA). Five VNTR loci were amplified in two different multiplex PCRs as described previously (12). The amplified samples were diluted 1:20 in water and 2 μl of each diluted sample was mixed with 18 μl of a 1:450 in water diluted GeneScan LIZ 500 size standard (Applied Biosystems, USA). After heat denaturation for 5 min at 95°C the fragments were separated with an ABI 3130 automated sequencer using the fragment analysis module. Sizing and calculation of the number of repeats of each VNTR were performed with GeneMarker software v1.80 (SoftGenetics, USA).

N. gonorrhoeae multiantigen sequence typing (NG-MAST). NG-MAST was performed as previously described (18,31). NG-MAST allele numbers of porB and tbpB, and sequence types (STs) were assigned using the NG-MAST database (www.ng-mast.net).

penA, mtrR, and porB1b (penB) sequencing. The penA gene, promoter region and the coding sequence of mtrR, and the full-length porB1b gene were sequenced as previously described (17). DNA of the N. gonorrhoeae WHO F reference strain and sterile, UV-treated water were included in all runs as positive and negative control, respectively (28).

Data analysis and statistics. Cluster analysis of the MLVA types were performed in Bionumerics Software v5.1 (Applied Math, Belgium). Statistical analyses were performed using SPSS v17.0 software (SPSS Inc., USA). Sequence alignments were performed using BioEdit v7.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).
RESULTS

Patient characteristics. Of the included patients, 155 (77%) were MSM, 30 (15%) heterosexual men, and 17 women (8%). The patients were predominantly of Dutch ethnicity (70%) and the median age was 35 years (range 27 to 43 years). Chlamydia trachomatis (31%) and HIV (31%) co-infections were common. Of eight (4%) patients (four women and four MSM), identified as commercial sex workers, five were from Eastern Europe (Table 1).

Antimicrobial susceptibility of all selected N. gonorrhoeae isolates. The MIC range of isolates with decreased susceptibility or resistance to cefotaxime were 0.125 to 0.50 µg/ml. No verified treatment failures were observed during the study period, however, these were not actively searched for. In vitro resistance to penicillin, tetracycline, and ciprofloxacin was common in groups A and B. In group B similar proportions of the isolates had resistance to these antibiotics, while the proportions in control group C were markedly lower (Table 2). Using an MIC of 0.016 µg/ml as an arbitrary cut-off 68% of the group A and 11% of group B isolates showed decreased susceptibility to cefixime and 76% and 57% showed decreased susceptibility to ceftriaxone, respectively. In contrast, 0 and 5% of control group C isolates showed decreased susceptibility to cefixime and ceftriaxone, respectively.

Detection of a penA mosaic allele in N. gonorrhoeae isolates. A penA mosaic allele was identified in 53 (26%) of the 202 examined N. gonorrhoeae isolates. The penA mosaic gene was exclusively found in isolates that had decreased susceptibility or resistance to cefotaxime. There was a strong positive correlation between N. gonorrhoeae isolates with higher cefotaxime MICs and the presence of a penA mosaic allele (Fig. 1). The frequency of the penA mosaic gene was significantly higher in group A (47/74, 64%) than in both group B (6/54, 11%) and group C (0/74) (p<0.0001, Chi²). Also the difference between group B and C was significant (p=0.0119, Chi²).
Cluster analysis of NG-MLVA profiles. To examine whether there was a clonal expansion of a penA mosaic positive N. gonorrhoeae strain within the Amsterdam high-risk population, all samples were genotyped with NG-MLVA. Hierarchical cluster analysis of the MLVA profiles of the 202 isolates identified two large clusters (≥10 isolates), that were assigned cluster I and II (Fig 2a).

NG-MLVA cluster I contained 56 isolates, including all 53 (95% of the cluster) penA mosaic-containing isolates. The remaining three were non-mosaic N. gonorrhoeae isolates that were susceptible to cefotaxime. In cluster II (n=39) no penA mosaic isolates were found.

The presence of cluster I, which included all 53 penA mosaic positive isolates with an identical penA mosaic allele (PBP2 pattern XXXIV), see below, strongly suggests the circulation of one clonal N. gonorrhoeae strain. A marked association was observed between NG-MLVA cluster I and decreased susceptibility and/or resistance to cefotaxime (95%). Most isolates in cluster I displayed in vitro resistance to tetracycline (89%) and ciprofloxacin (98%) according to EUCAST breakpoints (v1.3). In total, 118/128 isolates (92%) with decreased susceptibility to cefotaxime were also resistant to tetracycline and ciprofloxacin.

NG-MLVA cluster II may represent another group of clonally related strains, characterized by modestly increased MICs of cefotaxime. The proportion of isolates in the cefotaxime MIC groups A, B, and C in clusters I and II is illustrated in Figure 2b. Twenty-six (67%) of the isolates in cluster II (n=39) had a cefotaxime MIC of 0.125 µg/ml. Interestingly, 22/128 isolates (17%) with a cefotaxime MIC of ≥0.125 µg/ml could not be assigned to a large cluster.

Characteristics of the patients in NG-MLVA clusters I. The patients whose isolates comprised cluster I did not differ significantly from the total population regarding age, gender, sexual orientation, nationality, or co-infection status (Table 1). Of the patients in cluster I, 48 were MSM (86%), 5 heterosexual men (9%), and 3 women (5%). The patients in
cluster I were predominantly Dutch (71%) and co-infections with chlamydia (32%) and HIV (30%) were common. These characteristics were similar in cluster II. The only important difference between clusters I and II was the presence of seven (13%) patients with an Eastern European nationality who were only identified in cluster I. Of these, three (5%) patients were identified as commercial sex workers: one MSM (bisexual man) and two women. In total, four (7%) commercial sex workers were identified in cluster I while none of the patients in cluster II were commercial sex workers.

* N. gonorrhoeae multiantigen sequence typing (NG-MAST) of the isolates in NG-MLVA cluster I. On all 56 isolates of cluster I, NG-MAST was performed to confirm the clonality of the *N. gonorrhoeae* strain harboring a *penA* mosaic allele and to identify the NG-MAST sequence type(s) (ST) involved. Among these 56 isolates, 9 different NG-MAST STs were identified, two of which have not been previously described. Forty-six (87%) of the 53 *penA* mosaic positive isolates in cluster I were assigned to ST1407 and the remaining seven isolates were further differentiated in 5 other STs (Table 3). Compared to ST1407, four of these five STs only contained a single non-synonymous nucleotide substitution in *porB*, confirming closely related genotypes.

The remaining 3 non-mosaic *penA* isolates in cluster I had 3 different STs with multiple substitutions in both *porB* and *tbpB*, suggesting that these isolates contained more distantly related *N. gonorrhoeae* genotypes (Table 3).

*penA* sequences of the isolates within NG-MLVA cluster I. *PenA* sequencing was performed on all 56 isolates in NG-MLVA cluster I to confirm the identified *penA* mosaic positive gel patterns, determine the clonal relatedness of *penA*, and to identify the *penA* mosaic sequence type. Within these 56 isolates, all 53 *penA* mosaic positive isolates showed an identical *penA* mosaic sequence (PBP2 pattern XXXIV; GenBank accession no.
GU723422). In the remaining 3 non-mosaic penA isolates, the penA sequence pattern II was identified (Table 3).

Specific mutations in mtrR and porB1b (penB) in the isolates of NG-MLVA cluster I.

We examined two additional antibiotic resistance determinants that are associated with decreased ESC susceptibility. A specific nucleotide (A) deletion in the promoter region of the mtrR gene that causes over-expression of the MtrCDE efflux pump was observed in all 56 isolates of NG-MLVA cluster I. The remaining 3 non-mosaic isolates in NG-MLVA cluster I contained an additional G45D amino acid substitution in MtrR.

PorB1b amino acid substitutions G101K and A102N were detected in all but one penA mosaic positive isolates suggesting a decreased intake of the ESCs, while only one isolate had a wild type porB1b sequence. One mosaic and one non-mosaic isolate had a wild type porB1b sequence while the other two non-mosaic isolates had amino acid substitutions G101K and A102D (Table 3).

Genotypic characterization of a subset of isolates in cluster II. We genotypically characterized a subset of nine penA non-mosaic isolates from cluster II. Eight of the nine isolates had a cefotaxime MIC ≥0.125 µg/ml; four of these isolates had a borderline cefotaxime MIC of 0.125 µg/ml (group B) and four isolates had a cefotaxime MIC of 0.19 to 0.25 µg/ml (group A) and were considered resistant according to EUCAST (v1.3) resistance breakpoints. The remaining isolate that was included was susceptible to cefotaxime (group C) but had a ceftriaxone MIC of 0.023 µg/ml.

All nine non-mosaic penA isolates contained PBP2 pattern XII and the specific nucleotide (A) deletion in the promoter region of the mtrR gene. Eight of the nine isolates (including the group C isolate) contained porB1b alterations G101K/A102D and were assigned NG-MAST ST225. The remaining group A isolate had porB1b alterations G101K/A102N and was assigned NG-MAST ST5012.
DISCUSSION

Decreased *in vitro* susceptibility to extended-spectrum cephalosporins (ESCs), the last remaining treatment option for gonorrhea, is reported in many parts of the world. Due to the increasing number of reports concerning the loss of clinical treatment efficacy of orally administered ESCs, the parenteral ESC ceftriaxone is often considered to be the preferred first-line treatment for gonorrhea (7,25). However, a recent report described the first strain displaying high-level resistance to ceftriaxone (most likely related to a treatment failure using ceftriaxone) and complete characterization of this strain (20). It is now a fear that gonorrhea may become untreatable during certain circumstances and especially in some settings (20,25).

In the Netherlands, cefotaxime was used as first treatment option before 2006 due to non-availability of ceftriaxone in recommended dosages. From 2006 onwards, recommended dosages became available and ceftriaxone was recommended as first-line treatment for gonorrhea infections according to the national clinical guidelines which were issued by the Dutch Dermatological and Venereological Society (8). However, cefotaxime remained the drug of choice for monitoring ESC susceptibility to obtain comparable resistance data over a period of several years.

In the present study, the systematic monitoring of cefotaxime susceptibility from 2006 to 2008 allowed the detection of *N. gonorrhoeae* isolates with decreased susceptibility to ESCs among visitors of the STI outpatient clinic in Amsterdam (6). To assess these lower levels of antimicrobial resistance, *N. gonorrhoeae* isolates were classified according to the EUCAST resistance breakpoint for cefotaxime (MIC >0.125 µg/ml), rather than using the higher resistance breakpoint issued by the CLSI (MIC >0.5 µg/ml). Including *N. gonorrhoeae* isolates with borderline MICs of exactly 0.125 µg/ml enabled us to identify antimicrobial resistance determinants, that would have remained undetected when the EUCAST breakpoint for cefotaxime was strictly applied and thus only isolates with a MIC of >0.125 µg/ml were
included. In order to confirm whether cefotaxime is a good marker for susceptibility to extended-spectrum cephalosporins in general, we also recultured the *N. gonorrhoeae* strains and assessed their sensitivity to cefixime and ceftriaxone. Using an arbitrary breakpoint, we confirmed that decreased susceptibility to these other cephalosporins was frequently found in isolates with decreased susceptibility to cefotaxime, and not in control isolates.

Genotypic characterization of all of the included isolates showed the emergence of a *N. gonorrhoeae* strain, that harbored a *penA* mosaic allele (PBP2 pattern XXXIV), within the Amsterdam high-risk population, which has also been found in San Francisco (U.S.), Ontario (Canada), and Northern Taiwan (1,13,22). NG-MLVA revealed two large clusters (named NG-MLVA cluster I and II) and all *penA* mosaic positive isolates (n=53) were classified to cluster I (n=56). Additional NG-MAST analysis of the *penA* mosaic positive isolates in NG-MLVA cluster I revealed that ST1407 (87%) was predominant, whereas the remaining isolates contained some evolving but, to ST1407, very closely related subtypes. Three non-mosaic *penA* isolates (PBP2 pattern II), which were susceptible to ESCs, were also assigned to NG-MLVA cluster I, suggesting that these were more distantly related *N. gonorrhoeae* genotypes (12). The genotypic characterization of a limited number of isolates in cluster II predominantly revealed ST225, a worldwide-prevalent *N. gonorrhoeae* strain (9), that harbored a *penA* non-mosaic allele (PBP2 pattern XII).

The emergence and spread in many countries worldwide suggests ST1407 to be a *N. gonorrhoeae* strain that is successful in regard to transmission. The strain probably originated in the WHO Western Pacific Region and has acquired additional resistance determinants over time. Antimicrobial multi-resistant *N. gonorrhoeae* strains such as ST1407 may have a survival advantage in an antibiotic-rich environment, which is common in high frequency transmitting populations where many individuals are treated for various STIs on a regular basis (1,10,13,22,25,26,30). However, proof is lacking that this strain is indeed more
biologically fit. A well-designed and quality assured study that assesses the biological fitness
of such N. gonorrhoeae strain in vitro (e.g. various culture media) and in vivo (animal
models), including transformation experiments would be very valuable and provide more
insight in this matter (20). We assume that ST1407 was imported in the Dutch population,
although it is difficult to determine the exact time point of introduction. This assumption
might be supported by the fact that oral ESCs such as cefixime were never part of the
standard treatment regimen in the Netherlands, whereas ST1407 has shown a strong
association with decreased susceptibility and/or resistance to oral ESCs (10,22).

A significant association was observed between the presence of the penA mosaic allele and
the decreased susceptibility and/or resistance to cefotaxime. However, when the total number
of isolates with decreased susceptibility to cefotaxime (n=128) was taken into account, only
41% of these isolates contained the penA mosaic allele. Many of the non-mosaic isolates
found in NG-MLVA cluster II (n=39) had moderately increased MICs for cefotaxime. The
decreased susceptibility to cefotaxime in NG-MLVA cluster I was associated with the penA
mosaic allele in synergy with alterations in the mtrR promoter region and porB1b, whereas
the isolates in NG-MLVA cluster II lacked a penA mosaic allele. Although it is too early for
final conclusions since only a limited number of isolates in cluster II was characterized, the
data suggested that the moderately increased cefotaxime MICs were caused by the synergistic
behavior of sequential alterations in non-mosaic penA alleles, mtrR, porB1b, and possibly
unknown antimicrobial resistance determinants. As recent studies showed that penicillin
resistance determinants pilQ and ponA did not significantly affect susceptibility to ESCs,
other unknown alterations are likely involved in the development of decreased susceptibility
and resistance to ESC (10,15,17,34,38). However, accurate determination of the effect of
these individual resistant determinants on cefotaxime susceptibility is hindered by the penA
epistatic mutational effects, synergistic behavior of the identified antimicrobial resistance
determinants, and deficient knowledge concerning the unknown resistance factors involved. Accordingly, the clinical relevance of the moderately increased MICs for cefotaxime is still not clear.

The rapid increase in prevalence of the clonally related isolates suggests a continuous transmission of this ST1407 \textit{N. gonorrhoeae} strain among visitors of the Amsterdam STI clinic (6). The high number of MSM (86%) and the high frequency of coinfections that were found in NG-MLVA cluster I indicate that transmission was facilitated by high-risk sexual behavior that is characterized by numerous, often anonymous, sexual contacts over short periods of time. The identified commercial sex workers might have acted as a bridge group that could possibly explain the circulation of ST1407 among both heterosexuals and MSM. Prospective genotyping of isolates with decreased susceptibility and/or resistance to cefotaxime and other ESCs might provide further insight in gonococcal transmission patterns and the identification of potential transmission networks or core groups.

Special attention should be given to the clinical identification and treatment of pharyngeal gonorrhea, which is predominantly found among MSM and female sex workers. In the present study only 4.5% of strains originated from the pharynx. Pharyngeal gonorrhea is often asymptomatic and more difficult to treat (19,35). This reduced cure rate facilitates accumulation of genetic alterations and might cause the pharynx to act as a reservoir for gene transfer and recombination.

In conclusion, disturbing reports of cefixime and ceftriaxone treatment failure in well documented cases of urogenital and pharyngeal gonorrhea, respectively, might be the beginning of an impending threat towards ESC resistance and possibly untreatable gonorrhea in certain circumstances spreading worldwide (21,25,29,30,37). The findings of the present study underline the importance of continued surveillance of ESC susceptibility on a national and international level. However, serious exploration of alternative antimicrobial treatment
options as well as strategies, such as antimicrobial combination therapy, is essential as the development of new antimicrobial agents is hindered by declining investments and resistance to ESC spreading worldwide seems to be a matter of time.

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TABLE 1. Patient characteristics of all included patients (n=202) and of the patients that were assigned to large (≥10) NG-MLVA cluster I (n=56) and II (n=39).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total study population</th>
<th>Cluster I</th>
<th>Cluster II</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Patients (%)</td>
<td>202 (100)</td>
<td>56 (100)</td>
<td>39 (100)</td>
<td>107 (100)</td>
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<tr>
<td>Median (IQR) age (yr)</td>
<td>35 (27-43)</td>
<td>37 (29-44)</td>
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<td>34 (25-41)</td>
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<tr>
<td>Gender &amp; Sexual orientation</td>
<td></td>
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<tr>
<td>Men who have sex with men</td>
<td>155 (77)</td>
<td>48 (86)</td>
<td>36 (92)</td>
<td>71 (66)</td>
</tr>
<tr>
<td>Heterosexual men</td>
<td>30 (15)</td>
<td>5 (9)</td>
<td>2 (5)</td>
<td>23 (21)</td>
</tr>
<tr>
<td>Women</td>
<td>17 (8)</td>
<td>3 (5)</td>
<td>1 (3)</td>
<td>13 (12)</td>
</tr>
<tr>
<td>Nationality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dutch</td>
<td>142 (70)</td>
<td>40 (71)</td>
<td>31 (79)</td>
<td>71 (66)</td>
</tr>
<tr>
<td>Eastern European(^a)</td>
<td>14 (7)</td>
<td>7 (13)</td>
<td>0</td>
<td>7 (7)</td>
</tr>
<tr>
<td>Surinamese/Antillean</td>
<td>12 (6)</td>
<td>2 (4)</td>
<td>1 (3)</td>
<td>9 (8)</td>
</tr>
<tr>
<td>Other</td>
<td>34 (17)</td>
<td>7 (13)</td>
<td>7 (18)</td>
<td>20 (17)</td>
</tr>
<tr>
<td>Coinfections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlamydia trachomatis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV positive</td>
<td>63 (31)</td>
<td>18 (32)</td>
<td>12 (31)</td>
<td>33 (31)</td>
</tr>
<tr>
<td>Status unknown</td>
<td>63 (31)</td>
<td>17 (30)</td>
<td>14 (36)</td>
<td>32 (30)</td>
</tr>
<tr>
<td>Sex workers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>4 (2)</td>
<td>2 (4)</td>
<td>0</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Women</td>
<td>4 (2)</td>
<td>2 (4)</td>
<td>0</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

\(^a\) IQR, interquartile range.

\(^b\) Albania, Bulgaria, Hungary, Ukraine, Romania, Russia, and Czech Republic.

TABLE 2. Percentage of isolates with MICs above the susceptibility breakpoints of several antibiotics in cefotaxime group A, B, and C.

<table>
<thead>
<tr>
<th>Cefotaxime group (MIC in µg/ml)</th>
<th>Penicillin G</th>
<th>Tetracycline</th>
<th>Ciprofloxacin(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (&gt;0.125)</td>
<td>22%</td>
<td>80%</td>
<td>96%</td>
</tr>
<tr>
<td>B (0.125)</td>
<td>24%</td>
<td>74%</td>
<td>94%</td>
</tr>
<tr>
<td>C (&lt;0.125)</td>
<td>14%</td>
<td>35%</td>
<td>47%</td>
</tr>
</tbody>
</table>

\(^b\) Penicillin G, tetracycline, and ciprofloxacin resistance breakpoints (EUCAST v1.3).

\(^a\) Percentage of isolates with MICs above the CLSI ciprofloxacin resistance breakpoint (MIC ≥1 µg/ml) in cefotaxime group A, B, and C were 96%, 94%, and 54%, respectively.

TABLE 3. Phenotypic and genotypic characteristics of the isolates in NG-MLVA cluster I (n=56).

<table>
<thead>
<tr>
<th>Group</th>
<th>MIC (µg/ml)</th>
<th>NG-MAST</th>
<th>No. of isolates</th>
<th>Mosaic allele</th>
<th>PBP2 pattern</th>
<th>Polymorphisms in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>&gt;0.125</td>
<td>ST1407</td>
<td>41</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST3378</td>
<td>2</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST2212</td>
<td>1</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST420</td>
<td>1</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST3149</td>
<td>1</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST5013*</td>
<td>1</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td>B</td>
<td>0.125</td>
<td>ST1407</td>
<td>5</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST2212</td>
<td>1</td>
<td>Yes</td>
<td>XXXIV</td>
<td>promoter A deletion, K(^{H1}) N(^{W1})</td>
</tr>
<tr>
<td>C</td>
<td>&gt;0.125</td>
<td>ST437</td>
<td>1</td>
<td>No</td>
<td>II</td>
<td>promoter A deletion, G45D K(^{H1}) D(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST1466</td>
<td>1</td>
<td>No</td>
<td>II</td>
<td>promoter A deletion, G45D K(^{H1}) D(^{W1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST5011*</td>
<td>1</td>
<td>No</td>
<td>II</td>
<td>promoter A deletion, G45D WT WT</td>
</tr>
</tbody>
</table>

\(^*\) new NG-MAST sequence type
FIG 1. Association between the presence of a penA mosaic allele (n=53) and cefotaxime susceptibility.

FIG 2. (a) Minimum spanning tree of 202 N. gonorrhoeae isolates typed by NG-MLVA. Hierarchical cluster analysis of the MLVA DNA profiles was performed to display the genetic relationship between the MLVA types. Each circle represents an MLVA type and the size of each circle corresponds to the number of identical MLVA types it contains. MLVA types that contain the mosaic penA gene are represented by a purple circle. MLVA types that are connected by a thick solid line differ in 1 VNTR locus from each other, while MLVA types connected by a dotted line differ in 2 VNTR loci. A cluster was assigned when adjacent MLVA types did not differ more by than one VNTR locus and if a minimum of 10 MLVA types met this criterion. MLVA types that belong to a cluster are represented by a grey halo. Two large clusters (n≥10) were identified and assigned I (n=56) and II (n=39). (b) MLVA types that contain the mosaic penA gene are represented by the letter M (see also Fig 2a). MLVA types with a cefotaxime MIC of <0.125 µg/ml, 0.125 µg/ml, or >0.125 µg/ml are represented by a green, (group C), lilac (group B), or purple (group A) circle, respectively.