Emergence of a *Neisseria gonorrhoeae* clone with reduced cephalosporin susceptibility between 2014 and 2019 in Amsterdam, The Netherlands, revealed by genomic population analysis

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Background: Emerging resistance to cephalosporins in *Neisseria gonorrhoeae* (*Ng*) is a major public health threat, since these are considered antibiotics of last resort. Continuous surveillance is needed to monitor the circulation of resistant strains and those with reduced susceptibility.

Objectives: For the purpose of epidemiological surveillance, genomic population analysis was performed on *Ng* isolates from Amsterdam with a focus on isolates with reduced susceptibility to ceftriaxone.

Methods: WGS data were obtained from 318 isolates from Amsterdam, the Netherlands between 2014 and 2019. Isolates were typed according to MLST, *Ng* Multi-Antigen Sequence Typing (NG-MAST) and *Ng* Sequence Typing for Antimicrobial Resistance (NG-STAR) schemes and additional resistance markers were identified. Phylogenetic trees were created to identify genetic clusters and to compare Dutch and non-Dutch MLST7827 isolates.

Results: MLST7363 and MLST1901 were the predominant strains having reduced susceptibility to ceftriaxone during 2014–16; MLST7827 emerged and dominated during 2017–19. NG-STAR38 and NG-MAST2318/10386 were predominant among MLST7827 isolates. MLST7827 reduced susceptibility isolates carried a non-mosaic 13.001 *penA* allele with an A501V mutation and *porB1b* G120K/A121D mutations, which were lacking in susceptible MLST7827 isolates. Phylogenetic analysis of all publicly available MLST7827 isolates showed strong genetic clustering of Dutch and other European MLST7827 isolates.

Conclusions: MLST7827 isolates with reduced ceftriaxone susceptibility have emerged during recent years in Amsterdam. Co-occurrence of *penA* A501V and *porB1b* G120K/A121D mutations was strongly associated with reduced susceptibility to ceftriaxone. Genetic clustering of Dutch and other European MLST7827 isolates indicates extensive circulation of this strain in Europe. Close monitoring of the spread of this strain having an alarming susceptibility profile is needed.

Introduction

The emergence of resistance in *Neisseria gonorrhoeae* (*Ng*) poses a major public health threat. Current treatment recommendation is the last-resort extended-spectrum cephalosporin ceftriaxone, together with azithromycin as dual therapy.[1] Since the benefit of dual therapy is not evidence-based and emerging high-level azithromycin resistance has been found in many countries,[2] Dutch, French and UK treatment guidelines recommend ceftriaxone monotherapy. WHO guidelines endorse monotherapy as well, provided that local resistance data confirming

susceptibility to ceftriaxone are available.[1] However, single cases of ceftriaxone-resistant isolates have been reported over recent years, underlining the need for continuous surveillance of circulating strains.[3–6]

Although ceftriaxone-resistant isolates have only been reported in a few countries, a drift towards higher ceftriaxone MICs has been observed worldwide, indicating a global reduction in susceptibility.[7] Ceftriaxone resistance can be determined by mutations in several genes, such as *porB, ponA, mtrR, rpoB* and *rpoD*, but especially by A501 mutations or mosaicism in the *penA* gene.[8–11] Additional mutations outside mosaic *penA* genes might add to the resistant phenotype, although their effects remain to be proven.[8] Genotypic characterization of the ceftriaxone-resistant isolates found in Japan, France and the UK identified mosaic *penA* 37.001, 42.001 or 60.001 alleles in these isolates, which belonged to MLST7363/1901/1903 and *Ng* Multi-Antigen Sequence Type (NG-MAST) 4220/1407/3435.[3,4,6] A ceftriaxone-resistant isolate from Singapore belonged to different STs but contained the same mosaic *penA* 60.001 allele.[5]

Isolates belonging to MLST1901/NG-MAST1407 with mosaic *penA* alleles were highly prevalent among strains with reduced susceptibility to ceftriaxone also, suggesting that tracking this strain is most important for monitoring emerging resistance. However, Osnes *et al.*[12] recently reported the emergence of a strain with reduced susceptibility between 2016 and 2018 in Norway, belonging to MLST7827 and carrying a non-mosaic *penA* 13.001 allele with A501V mutation. They showed that this strain, with an alarming antimicrobial resistance profile, likely originated from Asia and potentially circulates in Europe. Analysing Dutch isolates with reduced susceptibility from 2009–17, De Laat *et al.*[13] found a shift from a mosaic *penA* allele towards a non-mosaic *penA* allele with A501 mutation. We have now further examined the genetic change among isolates with reduced susceptibility in Amsterdam. WGS data were used for genomic characterization isolates with reduced ceftriaxone susceptibility and a representative part of the susceptible gonococcal population isolated from 2014–19 in Amsterdam. We aimed to identify genomic characteristics associated with reduced susceptibility to ceftriaxone in the Amsterdam gonococcal population.

Methods

Isolate details and selection

Isolates were collected from *Ng*-positive visitors to the sexually transmitted infection (STI) outpatient clinic of the Public Health Service of Amsterdam. MICs of azithromycin, ciprofloxacin and ceftriaxone were routinely determined for all isolates using Etests according to the manufacturer's instructions (bioMérieux SA). Ciprofloxacin clinical breakpoints were determined according to EUCAST clinical breakpoints v11.0. For azithromycin, isolates with MIC < 0.5 mg/L were assigned as susceptible, MIC = 0.5 mg/L as intermediate and MIC ≥ 1.0 mg/L as resistant (epidemiological cut-off; ECOFF). For ceftriaxone, isolates with MIC ≤ 0.016 mg/L were assigned as susceptible, MIC = 0.023–0.064 mg/L as intermediate and MIC ≥ 0.094 mg/L as having reduced susceptibility or as resistant in the case of MIC > 0.125 mg/L. During the study period of January 2014 to July 2019, ceftriaxone-resistant isolates were not found among the 7323 isolates that were cultured and stored.

For genomic characterization of strains with reduced ceftriaxone susceptibility circulating in Amsterdam, all 82 isolates with ceftriaxone MIC ≥ 0.094 mg/L obtained during the study period were selected for WGS.

To characterize the gonococcal population circulating in Amsterdam, 244 isolates (3.4% of all available isolates) with ceftriaxone MIC < 0.094 mg/L obtained during the study period were also selected, resulting in a total selection of 326 isolates for WGS. Isolates were randomly selected after stratification on year of isolation and ceftriaxone MIC; for each reduced susceptibility strain, three isolates with MIC < 0.094 mg/L from the same year of isolation were randomly selected. Stratification on ceftriaxone MIC was done to get a distribution of MICs that were <0.094 mg/L in the selection, similar to the distribution of MICs that were <0.094 mg/L in the total Amsterdam gonococcal population.

DNA isolation and WGS

Selected isolates were taken from −80°C storage, grown overnight on chocolate blood agar plates and DNA was extracted from pure cultures. Isolates were sequenced on the Illumina MiSeq or Illumina NovaSeq 6000 platform (the latter was chosen for higher throughput). For Illumina MiSeq sequencing, DNA was extracted using isopropanol precipitation after lysis with NucliSENS easyMAG Lysis Buffer (bioMérieux SA) with glycogen (40 mg/L). The pellet was washed twice in 70% EtOH and dissolved in 50 μL of Tris-HCl at pH 8.0. DNA sequencing libraries were prepared with the KAPA HTP Library Preparation Kit (Roche Life Sciences) and Nextflex Dual-Indexed DNA barcodes (Bioo Scientific) and 300 bp paired-end sequenced. Regarding Illumina NovaSeq 6000 sequencing, DNA was extracted from harvested bacteria in DNA/RNA Shield buffer using the ZymoBIOMICS™ MagBead DNA Kit (ZYMO RESEARCH). DNA sequencing libraries were prepared with the Nextera XT DNA Library Preparation Kit with IDT for Illumina DNA/RNA UD Indexes (Illumina) and 150 bp paired-end sequenced. All raw reads are available in the European Nucleotide Archive under accession number PRJEB40983.

Bioinformatic analyses

Default settings were used unless noted otherwise. Raw sequence reads were filtered, trimmed and adapters were removed with fastp v0.20.0.[14] Reads were mapped to reference genome FA1090 (NC_002946.2) with BWA-MEM2 v2.2.1 to calculate coverage using the SAMtools package v1.11.[15,16] Isolates were excluded if coverage was <95%. Reads were assembled with Skesa v2.3.0 with a minimum contig length of 500 bp and assembly quality was assessed with QUAST v5.0.2.[17,18] For isolates with a total assembly length of >2.1 Mb, Kraken2 v2.0.8 was used to check for contamination.[19] Variants were called with Snippy v4.4.0 using reference genome FA1090 and a full core-genome alignment was created with the snippy-core option (https://github.com/tseemann/snippy). Gubbins v2.3.4 was used to identify regions of recombination in this alignment and to create a phylogenetic tree based on a recombination-filtered variant alignment, by using the general time-reversible model with gamma distribution (GTR-GAMMA) in RAxML v8.2.12.[20,21] The phylogenetic tree with metadata was visualized using iTOL and legends were added with PDF Pro.[22] Bayesian Analysis of Population Structure (BAPS) was performed using the rhierBAPS package v1.1.2 in R v3.6.3 [settings: maximum depth = 2; maximum number of populations (n.pops) = 75].[23]

Isolates were uploaded to the PubMLST database and MLST, NG-MAST and *Ng* Sequence Typing for Antimicrobial Resistance (NG-STAR) STs were extracted.[24,25] Novel MLSTs and NG-STAR STs were submitted to the PubMLST and NG-STAR databases, respectively. Annotation of resistance genes (*penA, porB, ponA, gyrA, parC*, 23S rRNA, *mtrA/R/C/D/E, rplD, rplV, rpmH, rpoB, rpoD*) was done using either the allele annotation available in the PubMLST database or by manually aligning allele sequences from PubMLST and identifying previously reported resistance mutations or mosaicism.[9,26–29] Raw reads were mapped against all 23S rRNA reference sequences in the PubMLST database to identify heterogeneous A2058/2059G/C2611T mutations in the four different alleles using Ariba v2.14.4.[30] Snakemake v5.6.0 was used for workflow

management.[31] The pipeline is freely available at https://github.com/jolin dadekorne/genomic-population-analysis-of-Neisseria-gonorrhoeae.

Comparison of MLST7827 isolates

The genetic relatedness of Dutch and non-Dutch MLST7827 isolates was assessed. PubMLST contains a total of 224 *Ng* isolates belonging to MLST7827 (August 2020), of which 63 are Dutch and 161 are non-Dutch isolates. For 14 non-Dutch isolates, only contigs were available and downloaded from PubMLST. For the other 147 non-Dutch isolates, raw sequence reads were downloaded. Subsequently, fastp was used for filtering, trimming and adapter removal. One Dutch MLST7827 isolate was hybrid assembled using Unicycler v0.4.8 with Illumina MiSeq and MinION Nanopore sequence data, yielding a circular chromosome.[32] Variants were called with Snippy v4.6.0 using the Dutch hybrid assembly as reference genome, either using raw reads or contigs with the –ctgs option. A recombination-corrected phylogenetic tree was created and visualized as described above. Median SNP distance per main genetic cluster was calculated using snp-dists v0.7.0 on the filtered variant alignment (https://github.com/tseemann/snp-dists).

Statistical analyses

Associations between patient and/or isolate characteristics were identified with two-tailed chi-squared or Fisher's exact tests using a 95% CI. The Bonferroni correction method was applied in the case of multiple testing. All statistical analyses were performed in R v3.6.3.

Ethics

According to the Dutch Medical Research Act Involving Human Subjects, no additional ethical approval was required for this study (W20_451 # 20.498).

Results

Sequencing data

Out of 326 isolates selected for WGS, 4 were excluded due to non-viable cultures, 1 due to >95% read contamination and 3 due to coverage of <95%. For the resulting 318 isolates, 252 441 reads were obtained on average per isolate, with an average coverage of 98.7% (Table S1, available as Supplementary data at *JAC* Online). The phylogenetic tree was created based on a recombination-filtered variant alignment of 18 683 sites.

Patient characteristics

The 318 isolates were derived from 314 patients: 8 isolates were obtained from 4 patients from two different anatomical locations. Isolates were mainly obtained from MSM (82%) and isolated from the anus (48%). The median patient age was 30 years and the majority of patients were aged between 24 and 34 years (50%) (Table 1).

Genomic epidemiology and characterization of resistance mutations

A midpoint-rooted phylogenetic tree was created based on the recombination-filtered variant alignment and two separate lineages were identified (Figure 1). The majority of isolates in the main lineage A (*n* = 216) were from patients reporting homosexual or bisexual intercourse (96%). Isolates from 2017–19 and isolates resistant to azithromycin, ciprofloxacin or ceftriaxone were significantly overrepresented in lineage A. Isolates in lineage B

Table 1. Patient and isolate characteristics

Patient characteristics	*N* = 314
Sex, *n* (%)	
male	286 (91)
female	28 (9)
Age, years	
median (range)	30 (16–65)
<24, *n* (%)	59 (19)
24–34, *n* (%)	159 (50)
35, *n* (%)	93 (30)
NA, *n* (%)	3 (1)
Sexual preference *n* (%)	
MSM	258 (82)
heterosexual	41 (13)
bisexual	12 (4)
NA, *n* (%)	3 (1)
Isolates sequenced	*N* = 318[a]
Year of isolation, *n* (%)	
2014–16	128 (40)
2017–19	190 (60)
Anatomical location *n* (%)	
Anus	152 (48)
Urethra	89 (28)
Vagina/cervix	14 (4)
Tonsil	62 (19.5)
Other	1 (0.5)

NA = not available.
[a]From four patients, two isolates were obtained from two anatomical locations.

(*n* = 102) were significantly associated with being female, aged <24 years and reporting heterosexual intercourse (Table S2).

BAPS clustering resulted in 14 clusters at level 1, of which 12 main clusters are visualized in Figure 1. MLSTs were determined for 317/318 (100%) isolates, yielding 56 different MLSTs, of which 26 were found for a single isolate. One isolate could not be assigned an MLST because of one incomplete locus. The 17 MLST clusters that contained ≥5 isolates were defined as main MLST clusters, with MLST7827 being the largest cluster, containing 63 isolates. MLST8135, MLST8163 and MLST11990 were only found in patients reporting heterosexual intercourse and MLST11990 was significantly associated with female patients (Table S3). NG-MAST and NG-STAR types were obtained for 318 (100%) and 304 (96%) isolates, respectively. Isolates not typable according to the NG-STAR scheme (*n* = 14) carried heterogeneous 23S rRNA alleles. The most prevalent NG-MAST and NG-STAR types in each MLST cluster are shown in Table 2.

Isolates belonging to MLST1901 were significantly overrepresented during 2014–16 (15/19) and none of these were isolated after 2018. Also, MLST7363 isolates were significantly overrepresented during 2014–16 (15/17). Remarkably, MLST7827 isolates were significantly overrepresented during 2017–19 (50/63), of which 41/50 (82%) were isolated during 2018–19 (Table S3). These results indicate a recent emergence of MLST7827, which
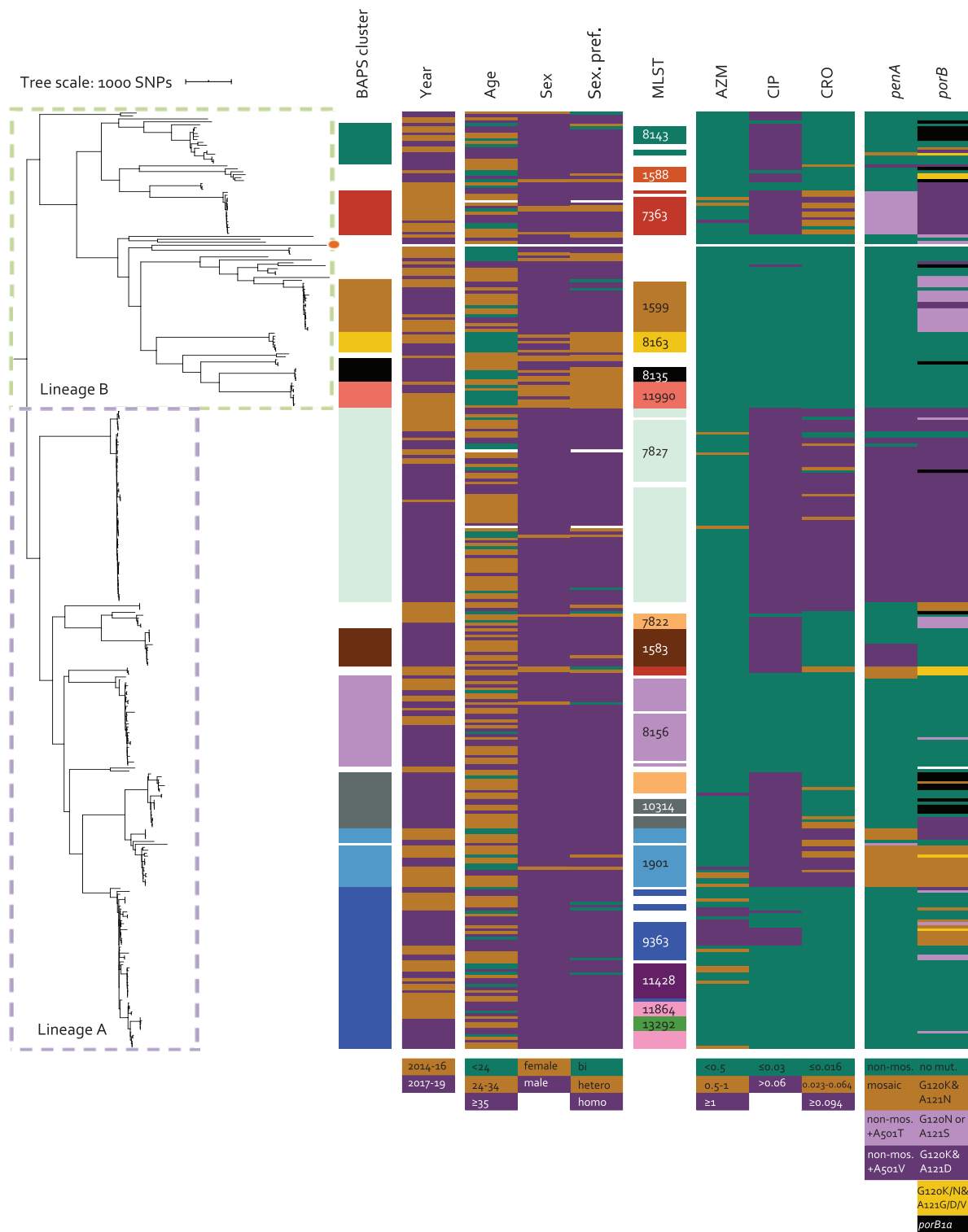
Figure 1. Recombination-filtered midpoint-rooted phylogenetic tree based on core-genome SNPs including 318 *Ng* isolates from 2014–19 from Amsterdam, the Netherlands. The FA1090 strain was used as the reference strain and its branch is visualized with an orange dot. Metadata includes: main clusters determined with BAPS analysis at level 1; year of isolation; patient characteristics (age, sex and sexual preference); MLST clusters containing ≥5 isolates; MICs in mg/L for azithromycin (AZM), ciprofloxacin (CIP) and ceftriaxone (CRO); *penA* type and *porB* type. *PorB1a* is given in black; all other colours represent different *porB1b* types. Phenotypic data are visualized as green for susceptible strains, orange for intermediate strains and purple for resistant strains or strains with reduced susceptibility. White bars indicate missing data. Two separate lineages are defined with dashed-line boxes.

Table 2. Most prevalent NG-MAST and NG-STAR types found in each MLST cluster

MLST cluster	Number of isolates	Main NG-MAST type (%)	Main NG-STAR type (%)
1583	13	15589 (92)	1340 (54)
1588	5	NA	NA
1599	17	11461 (65)	520 (76)
1901	19	1407 (58)	90 (32)
7363	17	2400 (35)	158 (53)
7822	12	14994 (33)	1387 (42)
7827	63	10368/2318 (35/33)	38 (89)
8135	5	387 (60)	729 (60)
8143	8	5624 (50)	426 (50)
8156	28	5441 (71)	442 (89)
8163	7	2 (29)	84 (86)
9363	18	12302 (28)	168 (28)
10314	9	NA	1387 (44)
11428	12	2992 (58)	63 (92)
11864	11	18234 (27)	439 (91)
11990	9	14376 (56)	962 (56)
13292	5	9208 (80)	439 (80)

NA, no dominant ST was found in that MLST cluster.

became the dominant strain with reduced ceftriaxone susceptibility in Amsterdam during 2017–19, instead of the previously dominating MLST1901 and MLST7363 strains.

Azithromycin

Azithromycin resistance was found for 15/318 (5%) isolates and resistance was significantly associated with MLST9363 (Table S3). Remarkably, a 23S rRNA C2611T mutation was only identified once in a susceptible isolate. Mosaicism in the *mtrR* promoter and gene was identified in 13/15 (87%) resistant isolates (Table 3). An additional fully mosaic *mtrC/D/E* operon was found in 12/13 (92%) and these mainly belonged to MLST9363 (9/12; 75%). The mosaic *mtrR* promoter and gene were only found in one susceptible isolate, belonging to MLST7367. The other two resistant isolates carried either a 35A deletion in the *mtrR* promoter or an *mtrR* A39T mutation and a non-mosaic or partly mosaic *mtrC/D/E* operon, but these alleles were also highly prevalent among susceptible isolates (Table 3). Mutations in *rplV, rmpH* and *mtrA* were not found at all (Table S1) and mutations in *rplD* and *mtrC* were not associated with resistance (Table 3).

Ciprofloxacin

Ciprofloxacin resistance was found for 173/318 (54%) isolates and significant associations were found with MLST1583, MLST1901, MLST7363 and MLST7827. Susceptibility was significantly associated with MLST1599, MLST8156, MLST11428, MLST11864 and MLST11990 (Table S3). All resistant isolates carried the *gyrA* S91F mutation (173/173), which was found in only 2/145 (1%) susceptible isolates, showing its importance in ciprofloxacin resistance. All isolates with an additional D95G mutation were resistant, whereas the two susceptible isolates carried a D95A/N mutation. *ParC*

D86N/S87/S88P mutations were highly prevalent among resistant isolates (147/173), although these are not required for resistance, given its absence in 15% of resistant isolates (Table 3).

Ceftriaxone

As a result of the selection strategy of this study, 80/318 (25%) isolates had reduced susceptibility to ceftriaxone and 26/318 (8%) had intermediate susceptibility. Intermediate and reduced susceptibility were significantly associated with MLST1901, MLST7827 and MLST7363 and susceptibility with MLST1599, MLST8156 and MLST9363 (Table S3). NG-MAST1407/NG-STAR90 was predominant among MLST1901 isolates, NG-MAST2400/NG-STAR158 among MLST7363 isolates and NG-MAST10386/NG-STAR38 and NG-MAST2318/NG-STAR38 among MLST7827 isolates (Table 2). Seventy-four percent (157/212) of the susceptible isolates carried a *porB1a* (20/157) or *porB1b* (137/157) gene without G120/A121 mutations and a non-mosaic *penA* gene without an A501 mutation (93%). The *porB1a* without G120/A121 mutations was only found in intermediate (1/21) or susceptible (20/21) isolates. *PorB1b* G120K and A121D/N mutations were found in 100% of the isolates with reduced susceptibility, 77% of the isolates with intermediate susceptibility and 10% of the susceptible isolates, indicating their importance in the resistance mechanism (Table 3). Nineteen percent (15/80) of the isolates with reduced susceptibility carried the mosaic *penA* allele 34.001 (11/15) or 10.001 (4/15) and belonged to MLST1901. Notably, these mosaic *penA* alleles were also found in intermediate MLST1901 (3/4) and MLST7363 isolates (2/11). Three susceptible isolates carried a mosaic *penA* allele, of which 63.001 and 92.001 were identified only once. In 42% of the isolates with intermediate susceptibility (11/26), either the non-mosaic *penA* allele 44.001 (10/11) or 18.001 (1/11) with A501T mutation was found, of which 82% (9/11) belonged to MLST7363. The majority of isolates with reduced susceptibility carried non-mosaic *penA* allele 13.001 with A501V mutation (72%), of which 97% (56/58) belonged to MLST7827 (Table 3, Figure 1). Three susceptible MLST7827 isolates were identified, of which one carried *porB1a* and the other two carried *porB1b* without G120/A121 mutations and non-mosaic *penA* without A501 mutation. *PorB1b* G120K/A121D mutations found outside the MLST7827 cluster and co-occurring with a non-mosaic *penA* allele without mutations were not associated with intermediate or reduced ceftriaxone susceptibility, except for three isolates in the MLST10314 cluster (Figure 1). However, intermediate or reduced susceptibility was observed outside the MLST7827 cluster when both *porB1b* mutations and either *penA* mosaicism or A501 mutations co-occurred (e.g. in MLST1901 and MLST7363 clusters). Moreover, isolates carrying a *penA* A501V mutation but lacking the *porB1b* mutations were susceptible (e.g. in the MLST1583 cluster). These findings show the interplay between mutations in *penA* and *porB* and that some of these mutations show a stronger effect on ceftriaxone MIC than others, thus gradually influencing the susceptibility. *PonA* L421P mutation, 35A deletion in the *mtrR* promoter and G45D in *mtrR* were found in the majority of intermediate and reduced susceptibility isolates. However, there was no direct association with reduced susceptibility since these mutations were also prevalent among susceptible isolates (Table 3). Mutations in *rpoB* and *rpoD* genes were not found (Table S1).

Table 3. Phenotypic characterization versus identified resistance mutations

Azithromycin	Susceptible (MIC < 0.5 mg/L) N = 289	Intermediate (MIC = 0.5 mg/L) N = 14	Resistant (MIC ≥ 1.0 mg/L) N = 15
23S rRNA (n = 318)[a]			
no A2058/A2059/C2611 mutations	288 (99.7)	14 (100)	15 (100)
C2611T in 1/4 alleles[b]	1 (0.3)		
mtrR promoter (−35A) and gene (A39T, G45D mutation) (n = 318)[a]			
no −35A/A39T/G45D mutations/ non-mosaic	35 (12.1)	—	—
−35A	66 (23)	5 (36)	1 (6.5)
−35A, A39T	1 (0.3)	—	—
−35A, G45D	68 (23.5)	3 (21)	—
mosaic promoter + gene	1 (0.3)	—	13 (87)
A39T	114 (39.4)	6 (43)	1 (6.5)
G45D	4 (1.4)	—	—
mtrC/D/E operon (n = 316)[a]			
non-mosaic	248 (86.5)	8 (57)	1 (6.7)
non-mosaic, GC del in *mtrC*	3 (1)	—	—
mosaic	1 (0.3)	—	12 (80)
partly mosaic	35 (12.2)	6 (43)	2 (13.3)
rplD (n = 318)[a]			
no G68/70 mutations	287 (99.3)	14 (100)	15 (100)
G70D	2 (0.7)	—	—

Ciprofloxacin	Susceptible (MIC ≤ 0.03 mg/L) N = 145		Resistant (MIC > 0.06 mg/L) N = 173
gyrA (n = 318)[a]			
no S91/D95 mutations	143 (99)		—
S91F, D95G	—		98 (57)
S91F, D95A	1 (0.5)		73 (42)
S91F, D95N	1 (0.5)		2 (1)
parC (n = 318)[a]			
no D86/S87/S88 mutations	140 (96.6)		26 (15)
D86N	—		85 (49)
S87I/N/R	4 (2.8)		60 (35)
S87R, S88P	1 (0.6)		2 (1)

Ceftriaxone	Susceptible (MIC ≤ 0.016 mg/L) N = 212	Intermediate (MIC 0.023–0.064 mg/L) N = 26	Reduced susceptibility (MIC ≥ 0.094 mg/L) N = 80
penA (n = 318)[a]			
non-mosaic/no A501 mutations	198 (93)	6 (23)	3 (4)
mosaic 34.001	—	3 (11.5)	11 (14)
mosaic 10.001	1 (0.5)	2 (8)	4 (5)
mosaic 63.001/92.001	2 (1)	—	—
non-mosaic 18.001 + A501T	—	1 (4)	—
non-mosaic 44.001 + A501T	1 (0.5)	10 (38)	4 (5)
non-mosaic 12.004 + A501V	—	1 (4)	—
non-mosaic 13.001 + A501V	2 (1)	3 (11.5)	58 (72)
non-mosaic 43.002 + A501V	8 (4)	—	—
porB (n = 317)[a]			
porB1a no G120/A121 mutations	20 (9)	1 (4)	—
porB1b no G120/A121 mutations	137 (65)	2 (8)	—
porB1b G120K, A121N	10 (5)	2 (8)	14 (17.5)
porB1b G120K, A121D	10 (5)	18 (69)	66 (82.5)

Continued

Table 3. *Continued*

Azithromycin	Susceptible (MIC < 0.5 mg/L) N = 289	Intermediate (MIC = 0.5 mg/L) N = 14	Resistant (MIC ≥ 1.0 mg/L) N = 15
porB1b G120N or A121S	29 (14)	—	—
porB1b G120K/N, A121G/D/V	5 (2)	3 (11)	—
mtrR promoter (−35A) and gene (A39T, G45D mutation) (n = 318)[a]			
no −35A/A39T/G45D mutations/ non-mosaic	33 (16)	2 (8)	—
−35A	37 (17)	13 (50)	22 (27.5)
−35A, A39T	—	1 (4)	—
−35A, G45D	9 (4)	4 (15)	58 (72.5)
mosaic promoter + gene	13 (6)	1 (4)	—
A39T	116 (55)	5 (19)	—
G45D	4 (2)	—	—
ponA (n = 318)[a]			
no L421P mutations	146 (69)	—	—
L421P	66 (31)	26 (100)	80 (100)

All shown as *n* (%).
[a]Gene in which mutations were identified (number of isolates this gene was characterized in).
[b]As determined by mapping raw reads against 23S rRNA reference sequences for identification of heterogeneous mutations.

Close genetic relatedness between Dutch and other European MLST7827 strains

The genetic relatedness of the 63 Dutch and 161 non-Dutch MLST7827 isolates publicly available in the PubMLST database was assessed. Available metadata showed that a large proportion of the non-Dutch MLST7827 isolates were from 2011–13 (43%) and from Asia (42%). The Dutch isolates from this study accounted for 28% of the MLST7827 isolates in the database. Regarding available phenotypic data, 100% (192/193) of the isolates were ciprofloxacin resistant but only 3% (3/119) were azithromycin resistant. Regarding ceftriaxone, 47% (91/195) showed reduced susceptibility and 1% (2/195) were resistant (Table 4).

Recombination-filtered variant alignment resulted in 7526 sites, on which the phylogenetic tree was based (Figure 2). The midpoint-rooted phylogenetic tree showed two lineages: main lineage A with three distinct clusters and lineage B with mainly Asian isolates from 2011–13. Clusters 1 and 3 mainly contained Dutch, Norwegian and European + UK isolates from 2014–19 and cluster 2 mainly contained Asian, but also American, Norwegian and European + UK isolates from 2011–13. Dutch isolates from 2017–19 were only found in cluster 1, together with Norwegian and European + UK isolates and one American isolate. The median SNP distances within clusters 1, 2 and 3 were 34, 89 and 33, respectively. This indicated stronger genetic relatedness among Dutch, Norwegian and European + UK isolates in clusters 1 and 3 than among isolates in cluster 2, which were mainly Asian. Cluster 1 contained most of the reduced ceftriaxone susceptibility isolates carrying non-mosaic *penA* 13.001 alleles with A501V mutation and *porB1b* G120K/A121D mutations. In contrast, lineage A mainly contained susceptible isolates carrying non-mosaic *penA* and *porB1a* alleles without mutations. Isolates in clusters 2 and 3 mainly carried non-mosaic *penA* 13.001 with A501V mutations; however, a variety of *porB1b* mutations were found in these

Table 4. Metadata available for *Ng* isolates belonging to MLST7827 obtained from the PubMLST database in August 2020

Number of MLST7827 isolates in PubMLST database	224	
Country/continent of isolation, *n* (%)		
Netherlands	63	(28)
Norway	30	(13)
Other European countries + UK	22	(10)
America (continent)	13	(6)
Asia	94	(42)
New Zealand	2	(1)
Year of isolation, *n* (%)		
2011–13	96	(43)
2014–16	45	(20)
2017–19	79	(35)
NA[a]	4	(2)
Ciprofloxacin MIC (mg/L), *n*		
≤0.03	1	
>0.06	192	
NA[a]	31	
Azithromycin MIC (mg/L), *n*		
<0.5	101	
0.5	15	
≥1.0	3	
NA[a]	105	
Ceftriaxone MIC (mg/L), *n*		
≤0.016	33	
0.023–0.064	69	
≥0.094–0.125	91	
>0.125	2	
NA[a]	29	

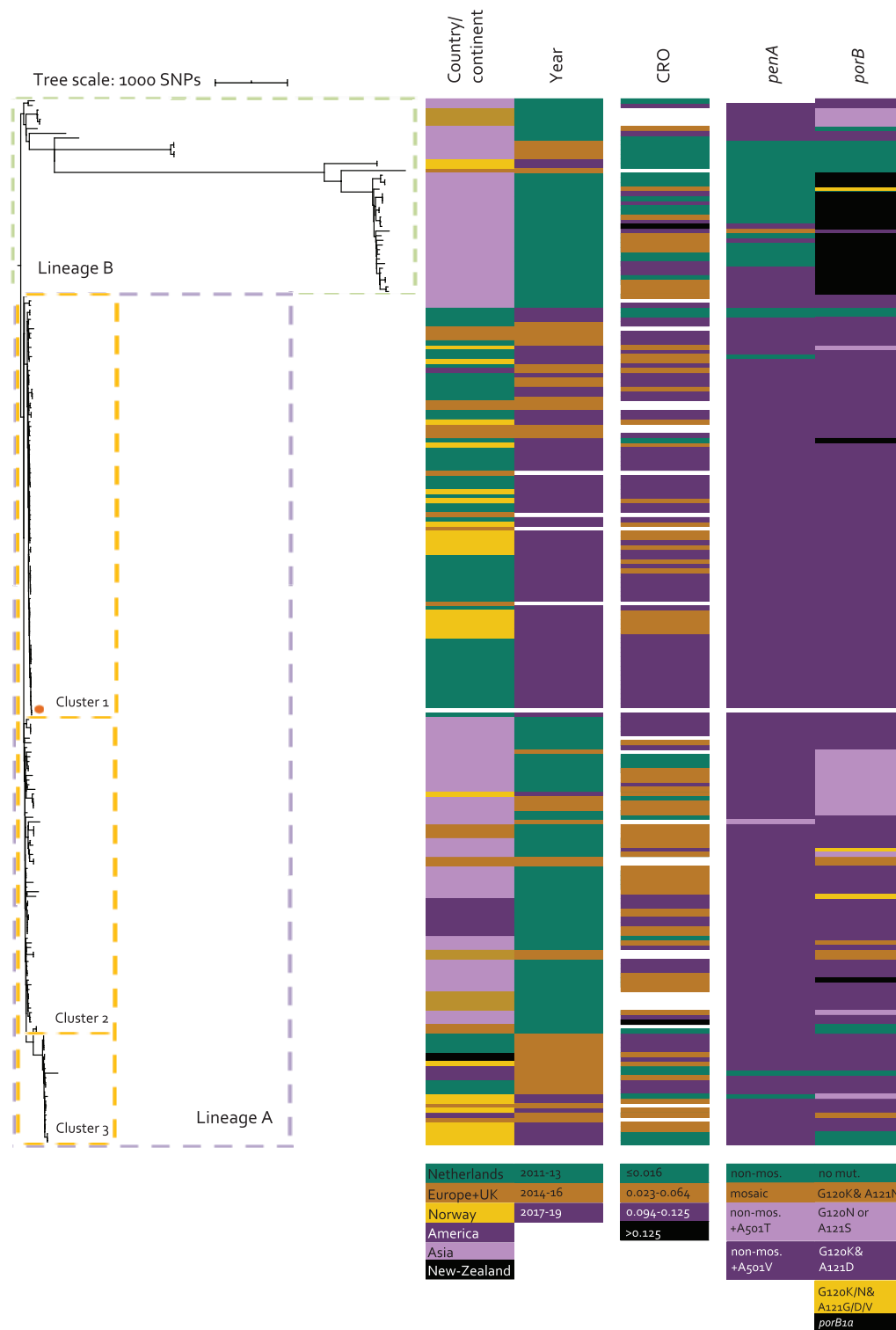[a]NA, data not available in PubMLST database.

Figure 2. Recombination-filtered midpoint-rooted phylogenetic tree based on core-genome SNPs including all 224 publicly available *Ng* isolates belonging to MLST7827. A Dutch MLST7827 isolate was used as the reference strain and its branch is visualized with an orange dot. Metadata includes: country/continent; year of isolation; ceftriaxone (CRO) MICs in mg/L are visualized as green for susceptible strains, orange for intermediate strains, purple for strains with reduced susceptibility and black for resistant strains; *penA* type; and *porB* type. *PorB1a* is given in black; all other colours represent different *porB1b* types. White bars indicate missing data. Dashed-line boxes define separate lineages and clusters.

clusters. Overall, reduced susceptibility to ceftriaxone was associated with the co-presence of the *penA* 13.001 A501V and *porB1b* G120/A121 mutations among global MLST7827 isolates. Metadata for the MLST7827 isolates are available in Table S4.

Discussion

This genomic population study extended the previous NG-MAST and *penA* typing study, which identified NG-MAST and *penA* shifts among *Ng* isolates with reduced ceftriaxone susceptibility from Amsterdam, obtained up until 2017.[13] Here we studied this phenomenon in more detail using WGS and including more recent isolates. The results showed that previous observations represented a shift from MLST1901 to MLST7363 and more recently to MLST7827. The emergence of the MLST7827 strain with reduced susceptibility to cephalosporins and resistance to ciprofloxacin in Amsterdam is in line with previously published surveillance articles. Peng *et al.*[33] reported MLST7827 as already being the predominant MLST in China during 2012–13, although at that time this MLST was not particularly associated with reduced susceptibility to cephalosporins. When this strain emerged in Norway during 2016–18 it was associated with reduced susceptibility to cephalosporins and we now confirm that the same emergence has occurred in the Netherlands during the last 3 years.[12] Since recent isolates from other European countries are scarce, these results can only suggest circulation of this strain in other parts of Europe.

Among the Dutch isolates, the co-occurrence of a non-mosaic *penA* 13.001 allele with A501V mutation and *porB1b* G120K/A121D mutations was associated with reduced susceptibility to ceftriaxone. Remarkably, these mutations were already found in isolates with reduced cephalosporin susceptibility from Korea during 2001–07, belonging to different NG-MAST STs.[34] From 2007 onwards, cephalosporin-resistant isolates belonging to MLST1901 or MLST7363 were identified that carried mosaic *penA* 37.001, 42.001 and 60.001 alleles; however, these *penA* alleles were not identified in this study. Other MLST and *penA* types have been found among isolates with reduced ceftriaxone susceptibility as well. In China, isolates belonging to MLST7363 and carrying a mosaic *penA* were responsible for reduced susceptibility during 2012–13.[33] We found both MLST1901 and MLST7363 to be associated with reduced susceptibility from 2014–16 in Amsterdam as well, confirming that these strains were the predominant strains with reduced susceptibility worldwide in previous years. In 2017, Abrams *et al.*[35] reported an isolate with reduced susceptibility that belonged to a different ST and lacked the mosaic *penA* allele. The *rpoB* and *rpoD* mutations found to be the genetic basis for reduced susceptibility in this isolate by Palace *et al.*[9] were not found among the Dutch isolates in the present study. Instead, reduced susceptibility was associated with MLST7827 isolates carrying *penA* and *porB1b* mutations, showing the interplay between these mutations in the resistance mechanism. This multifactorial nature also suggests additional and, as yet, unresolved genetic variations involved in cephalosporin resistance.

Previous research on Dutch isolates from 2008–15 showed a high prevalence of 23S rRNA mutations among azithromycin-resistant isolates with variable genetic backgrounds.[36] More recently, the influence of *mtr* mosaicism on azithromycin resistance has been described.[28] The results of this study suggest a replacement of mutations in 23S rRNA by mosaic *mtr* genes as the main determinant in azithromycin-resistant strains circulating in Amsterdam, using a limited number of azithromycin-resistant strains. Further research on larger numbers of azithromycin-resistant isolates is needed to confirm this observation.

Phylogenetic analysis of the 318 Dutch isolates revealed two separate lineages. Isolates in lineage A were significantly associated with bisexual or homosexual intercourse and resistance or reduced susceptibility to azithromycin, ciprofloxacin and ceftriaxone was significantly overrepresented. This lineage distinction was also seen in other isolate collections.[37] Previous studies state that MSM are more often infected with MDR isolates, probably because of the higher prevalence of bacterial STI in MSM. This leads to higher antibiotic exposure and increases selection pressure for antimicrobial resistance.[38]

Importantly, because of the MIC-based selection strategy, the percentage of isolates showing reduced ceftriaxone susceptibility in this study is not representative of the *Ng* population found among all STI clinic visitors in Amsterdam. Routine susceptibility testing showed that only 82 of 7323 (1.1%) isolates routinely obtained at the STI clinic had reduced ceftriaxone susceptibility during the study period. Although ceftriaxone-resistant isolates have not yet been found in the Netherlands, the emergence of MLST7827 isolates raises the question of whether this strain will evolve towards being a resistant strain. The two ceftriaxone-resistant MLST7827 isolates from China show the ability of this strain to become resistant according to the EUCAST clinical threshold, although these isolates did not cause therapy failure. High recombination rates in *Ng* enable the exchange of resistance mutations, which could cause a further reduction in susceptibility and ultimately lead to clinical resistance. Identification of the MLST7827 strain in multiple European countries over recent years shows its ability to spread quickly, underlining the need for global surveillance to track the prevalence and development of this strain.

Transparency declarations

None to declare.

Supplementary data

Tables S1 to S4 are available as Supplementary data at *JAC* Online.

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