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WGS analysis and molecular resistance mechanisms of azithromycin-resistant (MIC >2 mg/L) *Neisseria gonorrhoeae* isolates in Europe from 2009 to 2014

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Objectives: To elucidate the genome-based epidemiology and phylogenomics of azithromycin-resistant (MIC >2 mg/L) *Neisseria gonorrhoeae* strains collected in 2009–14 in Europe and clarify the azithromycin resistance mechanisms.

Methods: Seventy-five azithromycin-resistant (MIC 4 to >256 mg/L) *N. gonorrhoeae* isolates collected in 17 European countries during 2009–14 were examined using antimicrobial susceptibility testing and WGS.

Results: Thirty-six *N. gonorrhoeae* multi-antigen sequence typing STs and five phylogenomic clades, including 4–22 isolates from several countries per clade, were identified. The azithromycin target mutation A2059G (*Escherichia coli* numbering) was found in all four alleles of the 23S rRNA gene in all isolates with high-level azithromycin resistance ($n=4$; MIC ≥256 mg/L). The C2611T mutation was identified in two to four alleles of the 23S rRNA gene in the remaining 71 isolates. Mutations in *mtrR* and its promoter were identified in 43 isolates, comprising isolates within the whole azithromycin MIC range. No mutations associated with azithromycin resistance were found in the *rplD* gene or the *rplV* gene and none of the macrolide resistance-associated genes [*mef*(A/E), *ere*(A), *ere*(B), *erm*(A), *erm*(B), *erm*(C) and *erm*(F)] were identified in any isolate.

Conclusions: Clonal spread of relatively few *N. gonorrhoeae* strains accounts for the majority of the azithromycin resistance (MIC >2 mg/L) in Europe. The four isolates with high-level resistance to azithromycin (MIC ≥256 mg/L) were widely separated in the phylogenomic tree and did not belong to any of the main clades. The main azithromycin resistance mechanisms were the A2059G mutation (high-level resistance) and the C2611T mutation (low- and moderate-level resistance) in the 23S rRNA gene.

Introduction

The high global prevalence of gonorrhoea and the increasing antimicrobial resistance in *Neisseria gonorrhoeae* worldwide are major public health concerns.[1–3] Owing to the emergence of resistance in *N. gonorrhoeae* to ceftriaxone, which is the last option for empirical first-line gonorrhoea monotherapy, current treatment guidelines in Europe, Australia, USA and Canada recommend dual antimicrobial therapies, mainly 250–500 mg ceftriaxone intramuscularly as a single dose plus 1–2 g azithromycin

as a single oral dose.[4–9] However, azithromycin monotherapy is recommended as an alternative treatment regimen in several treatment guidelines, e.g. in European guideline (2 g single oral dose, in patients with known cephalosporin allergy when azithromycin susceptibility has been confirmed by laboratory susceptibility testing),[5] German guideline (1.5 g single oral dose, if *N. gonorrhoeae* strain is known to be susceptible)[9] and Canadian guideline (2 g single oral dose).[7] Furthermore, despite not being recommended, azithromycin monotherapy (1–2 g single oral dose) is still used in many countries (prescribed or delivered 'over-the-counter' without prescription) due to its wide availability, use for *Chlamydia trachomatis* infections and ease of administration. Unfortunately, resistance to azithromycin in *N. gonorrhoeae* has increased during the last decade in many geographic settings, and the resistance level in Europe has increased from 4.5% to 7.9% during 2012–14.[3,10–14] High-level resistance to azithromycin (MIC ≥256 mg/L) was initially verified in a *N. gonorrhoeae* isolate from 2001 in Argentina, was first detected in Europe in 2004 (in Scotland), and has since been reported in England, Ireland, Italy, Sweden, USA, Canada, China and Australia.[15–25]

The high use of azithromycin (in the treatment of gonorrhoea and infections caused by *C. trachomatis*, *Mycoplasma genitalium* and other bacteria) and the resulting increasing resistance in *N. gonorrhoeae* threatens the long-term sustainability of all currently available dual antimicrobial regimens for the treatment of gonorrhoea. Enhanced understanding of the international emergence and spread of azithromycin-resistant *N. gonorrhoeae* strains is imperative. For molecular epidemiology of *N. gonorrhoeae*, MLST and particularly *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) have been successfully used for many years.[26] In recent years, WGS has become more cost-effective, accessible and user-friendly. WGS with appropriate subsequent analysis provides an ideal solution for microepidemiology, macroepidemiology and identification of molecular antimicrobial resistance determinants.

Our aims were to elucidate the genome-based epidemiology and molecular mechanisms of azithromycin resistance (MIC >2 mg/L) in *N. gonorrhoeae* isolates ($n=75$) in the EU/European Economic Area from 2009 to 2014.

Materials and methods

The work was performed at the WHO Collaborating Centre for Gonorrhoea and other Sexually Transmitted Infections (Department of Laboratory Medicine, Microbiology, Örebro University Hospital, SE-701 85 Örebro, Sweden).

N. gonorrhoeae isolates

Seventy-five azithromycin-resistant clinical *N. gonorrhoeae* isolates from 17 countries (Figure 1) participating in the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) and, for quality control and comparisons, the two azithromycin-resistant WHO *N. gonorrhoeae* reference strains U and P[27] were examined. The Sweden/2012 clinical strain has also been subsequently assigned as a WHO reference strain (WHO V). The clinical *N. gonorrhoeae* isolates consisted of all isolates with an azithromycin MIC >2 mg/L reported within Euro-GASP from 2009 to 2012 ($n=66$) and nine additional isolates from 2013 and 2014 that also were submitted for the study. Euro-GASP has been previously described in detail.[11] In brief, participating laboratories submitted consecutive isolates for centralized susceptibility testing or, if laboratories fulfilled the quality assurance criteria,[11] they partook in decentralized testing. To represent different episodes of infection, laboratories were requested to collect only one isolate per patient from those who were infected multiple times within the requested 4 week period or at multiple sites; pharyngeal followed by rectal, then urethral specimens were preferred from males, and pharyngeal followed by cervical then other anogenital specimens from females. For centralized testing, isolates were tested at either PHE (London, UK) or Örebro University Hospital (Örebro, Sweden). For decentralized testing, laboratories performed their own antimicrobial susceptibility testing. All countries participating in Euro-GASP report the results, including associated patient epidemiological information, through The European Surveillance System (TESSy), a Web-based reporting system managed by the ECDC. According to EUCAST (www.eucast.org), *N. gonorrhoeae* isolates with an azithromycin MIC >0.5 mg/L should be categorized as resistant. However, in the present study only isolates with an azithromycin MIC >2 mg/L were selected for detailed examination because: (i) in repeated testing many of the isolates with an azithromycin MIC of 1–2 mg/L fluctuated between intermediate susceptibility and resistance; (ii) isolates with an azithromycin MIC of 1–2 mg/L were also a mixture of isolates with known molecular resistance mechanisms and isolates lacking resistance mechanisms; and (iii) the high number of isolates with an azithromycin MIC of 1 mg/L.

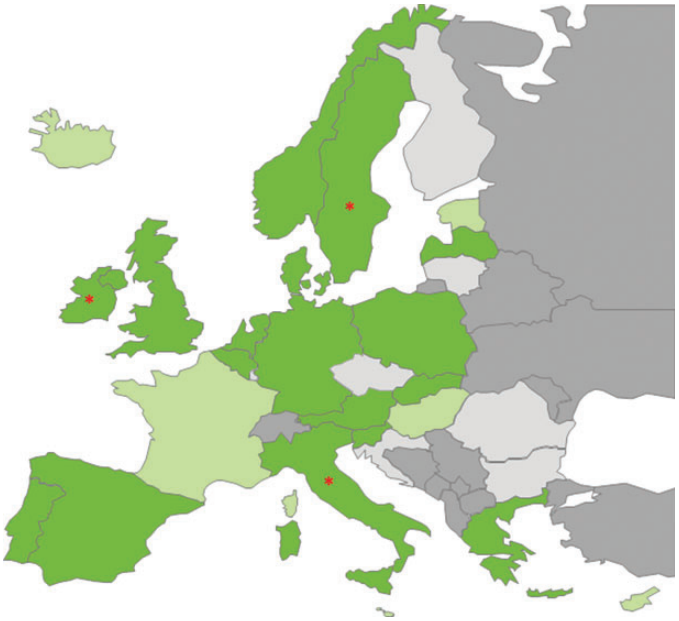

Figure 1. Azithromycin-resistant (MIC >2 mg/L) *N. gonorrhoeae* isolates ($n=75$) were collected from 2009 to 2014 in 17 (dark green) of the 23 countries participating in the Euro-GASP in 2014. The remaining six Euro-GASP countries are shown in light green, additional EU/European Economic Area (EEA) countries are shown in light grey and non-EU/EEA countries are shown in dark grey. Asterisks indicate isolates with MICs of azithromycin ≥256 mg/L during the study period. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Antimicrobial susceptibility testing

The MICs (mg/L) of azithromycin, ceftriaxone, cefixime, spectinomycin and ciprofloxacin were determined, with at least two replicates per isolate, using the Etest method (bioMérieux, AB, Solna, Sweden), in accordance with the manufacturer's instructions. MIC values were interpreted in

accordance with the clinical breakpoints stated by EUCAST (www.eucast.org). Only whole MIC dilutions were reported.

DNA extraction

DNA extraction was performed using a custom protocol on the QIAsymphony platform (QIAGEN GmbH, Hilden, Germany) using the QIAsymphony DSP Virus/Pathogen Midi Kit, Version 1 (QIAGEN). Briefly, the protocol was set to include an RNAse treatment step and to have a detergent-free elution buffer.

Genomic characterization

WGS was performed on all isolates using the Nextera XT DNA library preparation kit and the Illumina MiSeq platform, in accordance with the manufacturer's instructions. The sequencing yielded an average of 1 235 000 reads post-quality control and average genome coverage of 85×. Sequencing reads were assembled *de novo* using CLC Genomics Workbench 8.0.2 (https://www.qiagenbioinformatics.com/) with automatic bubble and word size.

Reads were aligned to the chromosome of the azithromycin-resistant reference strain WHO P[27] using SMALT (version 0.7.4) with GATK indel realignment. Variant sites were identified from each isolate using bcftools (version 0.19) within SAMtools (version 0.19)[28] and filtered as described previously[29] to produce a multiple sequence alignment. Phylogenetic analysis was performed using RAxML[30] and support was assessed via 100 bootstrap pseudoanalyses of the multiple sequence alignment. Recombinant regions were identified and removed using Gubbins[31] and a new maximum-likelihood phylogenetic tree based on vertically inherited SNPs was obtained. Phylogenomic clades were defined based on patristic distances in the phylogenetic tree using RAMI[32] identifying 10 clusters including two large clusters (>10 isolates) with a Shannon entropy[33] of 2.436 and evenness of 0.788.

NG-MAST alleles, MLST alleles and all macrolide molecular resistance determinants except 23S rRNA gene (see below) were identified *in silico* based on the *de novo* assemblies. The frequency of the 23S rRNA gene mutations were identified using the integrated mapping[34] and quality-based variant detection (Neighborhood Quality Standard algorithm)[35] within the CLC Genomic Workbench 8.0.2. The NG-MAST (http://www.ng-mast.net) and Neisseria MLST (http://pubmlst.org/neisseria) websites were used to assign allele numbers and STs. NG-MAST genogroups, comprising the main ST plus closely related STs, were assigned as previously reported.[36]

The macrolide molecular resistance determinants investigated included mutations in the peptidyltransferase region of domain V of the 23S rRNA gene, the *mtrR* repressor gene (including the promoter region and the mtr_{120} mutation), the *rplD* gene (encoding ribosomal protein L4) and the *rplV* gene (encoding ribosomal protein L22), as well as the possible presence of the *mef*(A/E) genes (encoding the Mef efflux pump), *ere*(A) and *ere*(B) genes (encoding erythromycin esterase) and *erm*(A), *erm*(B), *erm*(C) and *erm*(F) genes (encoding RNA methylases that block macrolides from binding to the 23S subunit target).[3,13,15,17,18,20–22,24,25,37–43]

Results

The phenotypic and genetic characterization of the azithromycin-resistant *N. gonorrhoeae* isolates are summarized in Table 1 and Figure 2.

Patient data

The *N. gonorrhoeae* isolates were collected from males (85%), females (11%) and from cases with gender not reported (4%). Among the male cases, 34% were reported as MSM. However,

Table 1. Country of isolation, patient data, MIC of azithromycin, molecular resistance determinants and NG-MAST of azithromycin-resistant (MIC >2 mg/L) *N. gonorrhoeae* isolates circulating in Europe from 2009 to 2014

MIC of azithromycin in mg/L (no.)	Country of isolation	Gender (no.)	Age in years, mean (range)	Infection site (no.)	Transmission (no.)	23S rRNA (no. of mutated alleles; no. of isolates)	*mtrR* (no.)	NG-MAST ST (no.)
4–8 (34)	Austria, Denmark, Germany, Greece, Ireland, Norway, Poland, Slovakia, Slovenia, Spain, The Netherlands, UK	M (30), F (4)	27 (18–58)	gen (30), rec (3), ph (1)	hetero (5), MSM (11), unk (18)	C2611T (2; 3), C2611T (3; 5), C2611T (4; 26)	A-del in prom (10), MtrR G45D (3), mtr_{120} (0)	2992 (7), 1407 (4), 1478 (4), 21 (2), 1313 (2), single STs (15)
16–32 (37)	Belgium, Denmark, Greece, Ireland, Italy, Latvia, Norway, Portugal, Slovenia, Spain, The Netherlands, UK	M (30), F (4), unk (3)	30 (17–70)	gen (32), rec (1), ph (1), unk (3)	hetero (9), MSM (10), unk (18)	C2611T (4; 37)	A-del in prom (24), MtrR G45D (2), mtr_{120}(0)	2992 (10), 5533 (6), 11463 (4), 2997 (2), 5108 (2), 10128 (2), single STs (11)
>256 (4)	Ireland, Italy, Sweden	M (4)	26 (20–49)	gen (3), rec (1)	hetero (2), MSM (1), unk (1)	A2059G (4; 4)	A-del in prom (2), MtrR G45D (2), mtr_{120} (0)	649 (2), 8927 (1), 4980 (1)

No., number; M, male; F, female; gen, genital; rec, rectal; ph, pharyngeal; hetero, heterosexual; unk, unknown; A-del in prom, deletion of A in the 13 bp inverted repeat sequence of the *mtrR* promoter.

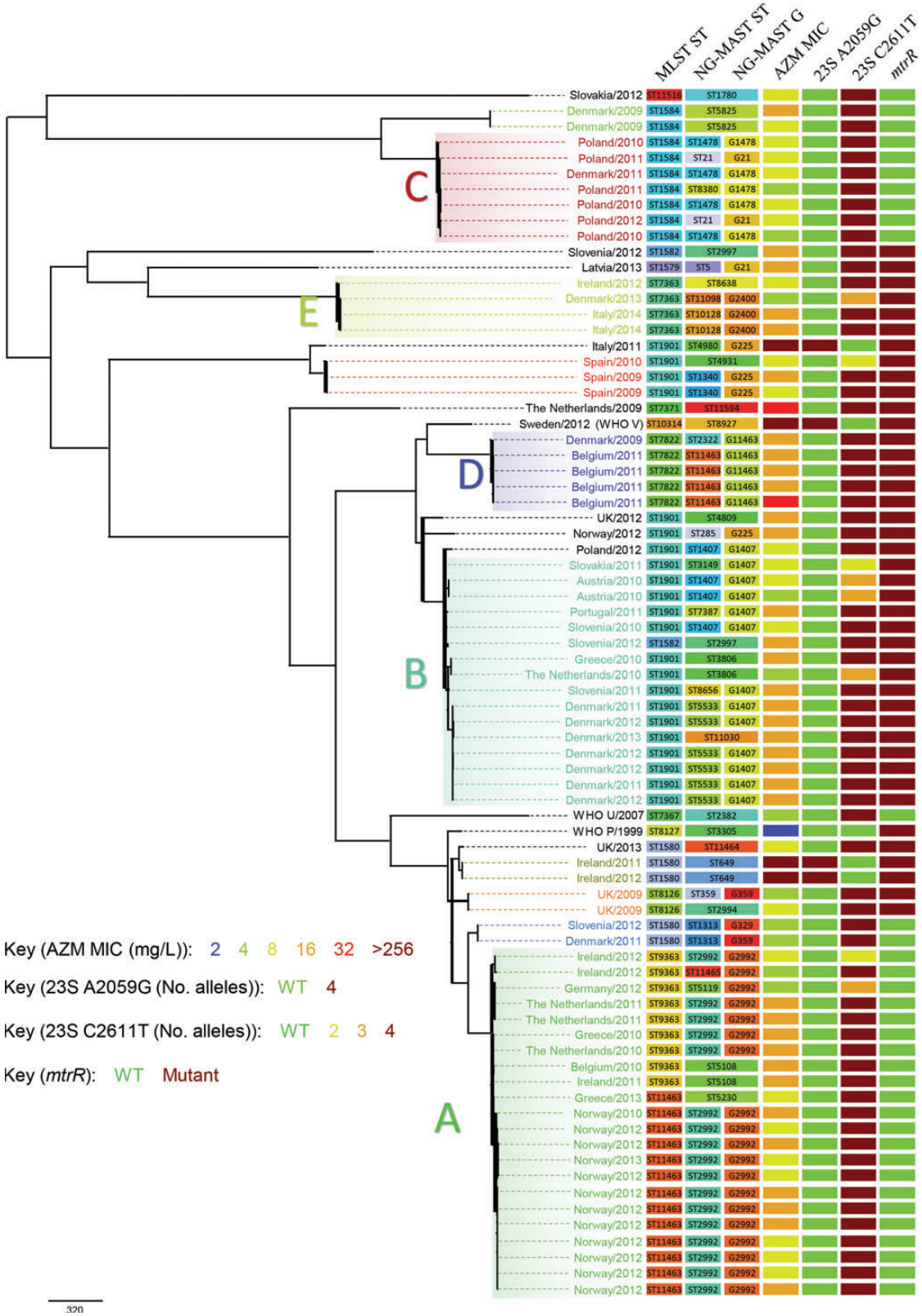

Figure 2. Whole core genome SNP maximum likelihood phylogenetic tree of 75 AZM-resistant (MIC >2 mg/L) *N. gonorrhoeae* isolates cultured from 2009 to 2014 in 17 European countries (country and year indicated). Scale bar represents 320 SNPs when reconstructing the tree after recombinant regions were removed using Gubbins.[31] MLST, *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) ST and genogroup (G), AZM MIC and

the mode of sexual transmission was not reported in 47% of the male cases. The median age of all patients, and male and female patients was 28 years (range: 17–70 years), 28 years (range: 17–70 years) and 28 years (range: 18–58 years), respectively. Site of infection was genital (87%), rectal (7%), pharyngeal (3%) and not reported (4%).

Antimicrobial susceptibility

Thirty-four (45%) isolates displayed low-level azithromycin resistance with MICs of 4–8 mg/L, 37 (49%) displayed moderate-level azithromycin resistance with MICs of 16–32 mg/L and four (5.3%) displayed high-level azithromycin resistance with MICs of >256 mg/L (Table 1 and Figure 2). The four isolates with high-level azithromycin resistance were cultured in Ireland ($n=2$; 2011 and 2012), Italy (2011) and Sweden (2012), and all these isolates were susceptible to ceftriaxone (MICs of 0.016–0.032 mg/L) and spectinomycin, and obtained from males [urogenital heterosexual ($n=2$), anorectal MSM ($n=1$) and non-reported sexual transmission ($n=1$)]. Thirty-three (44%) isolates were additionally resistant to ciprofloxacin and seven (9.3%) isolates displayed cefixime resistance. All isolates were susceptible to spectinomycin and ceftriaxone; however, one from Austria displayed a ceftriaxone MIC of 0.125 mg/L that is exactly at the breakpoint for resistance.

Molecular macrolide resistance determinants

The azithromycin target mutation A2059G (*Escherichia coli* numbering; A2045G in *N. gonorrhoeae* NCCP11945) was found in all four alleles of the 23S rRNA gene in all the isolates with high-level resistance to azithromycin ($n=4$), but not in any of the other isolates ($n=71$). The C2611T mutation (*E. coli* numbering; C2597T in *N. gonorrhoeae* NCCP11945) was identified in the 23S rRNA gene in the remaining 71 isolates. Accordingly, the C2611T mutation was present in all four 23S rRNA gene alleles in 63 of the isolates, comprising all 37 isolates with an azithromycin MIC of 16 or 32 mg/L, and in two ($n=3$) or three ($n=5$) alleles of the 23S rRNA gene in eight isolates, all with an azithromycin MIC of 4 or 8 mg/L. The four isolates with high-level resistance to azithromycin did not contain the C2611T mutation (Table 1 and Figure 2).

The −35A deletion in the inverted repeat sequence in the promoter region of the *mtrR* gene was identified in 36 isolates and the G45D mutation in MtrR was found in seven isolates. These isolates comprised those within the whole azithromycin MIC range identified, i.e. from 4 to >256 mg/L (Table 1 and Figure 2). The mtr_{120} mutation was not found in any of the 75 isolates.

The majority of isolates had an identical *rplD* gene sequence ($n=43$); however, 26 contained V125A, A147G and R157Q mutations and six contained a G68D mutation. Nevertheless, when excluding other macrolide resistance determinants none of these mutation patterns appeared to be associated with an increased MIC of azithromycin. No point mutations were found in the *rplV* gene.

None of the macrolide resistance-associated genes [*mef*(A/E), *ere*(A), *ere*(B), *erm*(A), *erm*(B), *erm*(C) and *erm*(F)] was identified among the isolates.

MLST, NG-MAST and phylogenomic analysis

Using MLST, 13 different STs were revealed among the 75 azithromycin-resistant *N. gonorrhoeae* isolates. Most frequent STs were ST1901 ($n=22$), ST11463 ($n=13$), ST1584 ($n=9$) and ST9363 ($n=9$). NG-MAST identified 36 different STs among the isolates. ST2992 predominated ($n=17$), followed by ST5533 ($n=6$), ST1407 ($n=4$), ST1478 ($n=4$) and ST11463 ($n=4$), and 22 STs represented by single isolates were found (Table 1 and Figure 2). Five NG-MAST genogroups comprising four or more isolates, G2992 ($n=19$), G1407 ($n=13$), G1478 ($n=5$), G11463 ($n=5$) and G225 ($n=4$), were defined, which encompassed 46 (61%) of the 75 azithromycin-resistant *N. gonorrhoeae* isolates.

By phylogenomic analysis, 10 clades with two or more isolates, including two large clades (16–22 isolates), were identified. Fifty-four (72%) isolates were grouped into five clades (A–E) that contained from 4 to 22 isolates each (Figure 2). Clade A ($n=22$) comprised isolates belonging to MLST ST11463 (59%) or ST9363 (41%), and mainly NG-MAST G2992 (86%). The isolates contained the C2611T mutation in two (4.5%), three (4.5%) or four (91%) alleles of the 23S rRNA gene and a WT *mtrR* gene, including its promoter region (100%). The isolates in clade A had an azithromycin MIC of 4–16 mg/L and the majority (55%) of isolates was cultured in Norway in 2010–13, but isolates obtained in five additional countries in 2010–13 were also included in this clade. Clade B ($n=16$) contained isolates belonging to MLST ST1901 (94%) or ST1582 (6%), and were mostly (75%) assigned as NG-MAST G1407. The majority (75%) of isolates contained the C2611T mutation in all four alleles of the 23S rRNA gene, and the remaining isolates had two (6%) or three (19%) 23S rRNA alleles with the C2611T mutation. All isolates had the −35A deletion in the *mtrR* promoter region and one isolate additionally contained the G45D mutation in MtrR. Isolates in clade B displayed an azithromycin MIC of 4–16 mg/L and 44% of the isolates were obtained in Denmark (2011–13), but isolates from six additional countries were also in this clade. Clade C ($n=7$) contained isolates belonging to MLST ST1584, and NG-MAST G1478 (71%) or G21 (29%). All these isolates possessed the C2611T mutation in all four alleles of the 23S rRNA gene and a WT *mtrR* gene, including its promoter region. The isolates displayed an azithromycin MIC of 4 or 8 mg/L and were cultured in Poland (86%) or Denmark (14%) in 2010–12. The isolates in clade D ($n=5$) belonged to MLST ST7822 and NG-MAST G11463. All these isolates had the C2611T mutation in all four alleles of the 23S rRNA gene and the −35A deletion in the *mtrR* promoter

resistance mutations in the 23S rRNA gene and the *mtrR* promoter (−35A deletion in the inverted region), are indicated. When the assigned NG-MAST ST did not belong to any NG-MAST genogroup, the bar covers both of these fields. For the AZM MIC and the molecular resistance determinants, green segments represent lower AZM MIC and absence of the particular resistance determinant (WT), respectively. Isolates of each of the 10 identified clades are indicated in an identical colour and heterogeneous isolates outside these clades are indicated in black. Two AZM-resistant WHO reference strains (WHO P and U) were included for comparison. The Sweden/2012 strain has also been subsequently assigned as a WHO reference strain (WHO V). AZM, azithromycin.

region. The isolates had an azithromycin MIC of 16 or 32 mg/L and were obtained in Belgium (80%; 2011) or Denmark (20%; 2009). Finally, clade E isolates ($n=4$) belonged to MLST ST7363 and mainly (75%) NG-MAST G2400. All these isolates contained the C2611T mutation in three (25%) or four (75%) alleles of the 23S rRNA gene and the −35A deletion in the *mtrR* promoter region. The isolates had an azithromycin MIC of 4–16 mg/L and were cultured in Denmark, Ireland and Italy in 2012–14. The four isolates with high-level resistance to azithromycin (MIC ≥256 mg/L) were, genomically, widely divergent, did not belong to any of the main clades, and were assigned as MLST ST1580 (NG-MAST ST649), ST1901 (ST4980) and ST10314 (ST8927), respectively (Figure 2).

Discussion

The increasing resistance to azithromycin in *N. gonorrhoeae* internationally threatens the long-term sustainability of the recommended first-line dual antimicrobial regimens for treatment of gonorrhoea. In the present study, we describe the molecular epidemiology, using phylogenomics, NG-MAST and MLST, of azithromycin-resistant *N. gonorrhoeae* strains circulating in 2009–14 in Europe and elucidate the genetic mechanisms resulting in the azithromycin resistance in these strains. It is a major concern that the resistance to azithromycin in *N. gonorrhoeae* has increased during the last decade in many geographic settings, and the resistance level in the Euro-GASP countries has increased from 4.5% to 7.9% during 2012–14.[3,10–14] Our results show that many azithromycin-resistant *N. gonorrhoeae* strains were transmitted in Europe. However, five phylogenomic clades (4–22 isolates per clade) containing 54 (72%) of the 75 azithromycin-resistant isolates were identified, which shows that the clonal spread of relatively few *N. gonorrhoeae* strains accounted for the majority of the azithromycin resistance and these strains were found in 13 of the Euro-GASP countries. The identification of several major clades of azithromycin-resistant *N. gonorrhoeae* is also in line with a recent publication from Canada.[13] However, the four isolates with high-level resistance to azithromycin (MIC ≥256 mg/L), cultured in Ireland ($n=2$), Italy ($n=1$) and Sweden ($n=1$), were widely divergent, did not belong to any of the main clades and, represented three different emergences of high-level azithromycin resistance. These results together with previous publications[15–25] elucidate that many different *N. gonorrhoeae* strains, independent of their genomic background, have the potential to develop high-level resistance to azithromycin, i.e. by acquisition/selection of only one specific SNP (A2059G) in three to four alleles of the 23S rRNA gene. A major concern is that the azithromycin resistance in *N. gonorrhoeae* appears to be increasing rapidly in many countries,[3,10–14] which might be affected by inappropriate azithromycin use in monotherapy (for gonorrhoea, but also other bacterial sexually transmitted infections such as chlamydial and *M. genitalium* infections), but also use in the implemented dual antimicrobial therapies. This selective pressure of azithromycin might induce/select SNPs in one or several alleles of the 23S rRNA gene and, particularly under continuous macrolide selective pressure, endogenous homologous recombination of the mutated 23S rRNA allele(s) might rapidly result in the presence of four mutated 23S rRNA alleles.[17] However, at least one *N. gonorrhoeae* strain or genomic lineage with high-level azithromycin resistance appears to also have clonally spread internationally over many years. In fact, both of the high-level azithromycin-resistant *N. gonorrhoeae* isolates cultured in Ireland in the present study belonged to NG-MAST ST649 and *N. gonorrhoeae* isolates assigned as NG-MAST ST649 or genetically closely related STs (belonging to genogroup 649)[36] with high-level resistance to azithromycin have been identified during the recent decade in Scotland,[16] Ireland,[18] England and Wales,[17,25] Australia[24] and the USA.[21] In 2015, an ongoing outbreak of high-level azithromycin-resistant gonorrhoea in England, due to a NG-MAST genogroup 649 (ST9768) strain, was described.[25]

In the present study, it was also elucidated that the A2059G mutation (*E. coli* numbering) in all four alleles of the 23S rRNA gene, resulting in a decreased affinity for the 23S rRNA target,[3] caused the azithromycin resistance in the four *N. gonorrhoeae* strains with high-level azithromycin resistance (MIC >256 mg/L), which is in concordance with previous findings.[13,15,17,18,20–22,24,25,43] The A2059G mutation was not present in any other isolates. Furthermore, all isolates with a moderate level of resistance to azithromycin (MIC of 16 or 32 mg/L) contained the C2611T mutation (*E. coli* numbering) in all four alleles of the 23S rRNA gene and isolates with low-level azithromycin resistance (MIC of 4 or 8 mg/L) contained the C2611T mutation in two or three 23S rRNA gene alleles. Mutations in the *mtrR* gene or its promoter region, resulting in an increased efflux of azithromycin through an overexpressed MtrCDE efflux pump,[3,40,41] were mainly randomly distributed among all isolates. Accordingly, in *N. gonorrhoeae* isolates with higher MICs of azithromycin, e.g. due to mutations in the 23S rRNA gene, the mutations in *mtrR* and its promoter did not significantly affect the MIC of azithromycin. Furthermore, no mutations associated with azithromycin resistance were found in the *rplD* gene or the *rplV* gene and none of the macrolide resistance-associated genes [*mef*(A/E), *ere*(A), *ere*(B), *erm*(A), *erm*(B), *erm*(C) and *erm*(F)] were identified in any of the examined azithromycin-resistant (MIC >2 mg/L) *N. gonorrhoeae* isolates in Europe in 2009–14. All these results suggest that future assays for molecular prediction of clinical resistance to azithromycin in *N. gonorrhoeae* should focus primarily on detection of the A2059G and C2611T mutations in the 23S rRNA gene. However, the effects of other previously described and new emerging macrolide resistance determinants should not be neglected and continuously monitored closely.

In general, despite WGS providing, as expected, an increased resolution between isolates, MLST and NG-MAST (particularly using genogroups) correlated relatively well with the phylogenomic clades. However, isolates belonging to the identical phylogenomic clade could be assigned as different MLST STs and particularly NG-MAST STs, which can significantly confuse the interpretation of the results when these methods are used. Notably, the phylogenomic clades A, B and E of azithromycin-resistant *N. gonorrhoeae* isolates in Europe appeared to correspond to phylogenomic clades A, H and L, respectively, in a recent publication investigating azithromycin-resistant *N. gonorrhoeae* isolates in Canada.[13]

The currently used dual antimicrobial treatment regimens (ceftriaxone plus azithromycin)[4–9] for gonorrhoea appear to be highly effective. However, it cannot be excluded that the use of, in particular, 1 g (but also 2 g) of azithromycin in the dual antimicrobial treatment regimens might additionally contribute to the selection of high-level azithromycin-resistant (MIC ≥256 mg/L) *N. gonorrhoeae* strains,

which have been most prevalent in the UK where 500 mg ceftriaxone plus 1 g azithromycin is recommended.[16,17,25] Unfortunately, it is likely that treatment failures with all of the currently available dual antimicrobial treatment regimens will emerge. This is because susceptibility to ceftriaxone in *N. gonorrhoeae* has decreased globally, azithromycin resistance is relatively prevalent in many countries, and concomitant resistance to ceftriaxone and azithromycin has also been identified in several countries.[3,9,10,13,14,22,44] In the present study, all azithromycin-resistant (MIC >2 mg/L) *N. gonorrhoeae* isolates were susceptible to ceftriaxone; however, one isolate from Austria displayed a ceftriaxone MIC of 0.125 mg/L, which is exactly at the breakpoint for resistance. In addition, seven (9.3%) azithromycin-resistant isolates with concurrent resistance to cefixime (MIC >0.125 mg/L) were identified (in clade B) and, in total, 13 isolates were assigned as MLST ST1901 and NG-MAST G1407, which is the internationally reported *N. gonorrhoeae* clone that has accounted for most of the resistance to extended spectrum cephalosporins throughout the world.[3,9,36] Finally, dual antimicrobial therapy regimens will not be affordable in many countries globally and, consequently, it is essential to develop novel antimicrobials for effective treatment of gonorrhoea.

In conclusion, enhanced phenotypic and molecular understanding of the international emergence and spread of *N. gonorrhoeae* strains resistant to different antimicrobials, including azithromycin, is imperative. This knowledge might give us the opportunity to counteract this evolution and spread and, at a minimum, to develop rapid and reliable genetic methods for prediction of the antimicrobial resistance, including directly in *N. gonorrhoeae* clinical samples for molecular diagnostics. In the present study, it was shown that the clonal spread of relatively few *N. gonorrhoeae* strains accounted for the majority of the azithromycin resistance (MIC >2 mg/L) in 2009–14 in Europe. The main genetic resistance mechanisms were the A2059G mutation (resulting in high-level azithromycin resistance) or the C2611T mutation (resulting in low- and moderate-level azithromycin resistance) in the 23S rRNA gene. WGS can revolutionize both the understanding of the molecular epidemiology of *N. gonorrhoeae* and the genetic prediction of antimicrobial resistance in *N. gonorrhoeae*. WGS might also provide an ideal solution for microepidemiology, macroepidemiology and public health information and interventions, i.e. in combination with antimicrobial resistance data and epidemiological, including sexual behaviour, data of the patients.

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Transparency declarations

None to declare.

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