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ORIGINAL ARTICLE

Antimicrobial resistance and molecular epidemiology using whole-genome sequencing of *Neisseria gonorrhoeae* in Ireland, 2014–2016: focus on extended-spectrum cephalosporins and azithromycin

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Abstract

High-level resistance and treatment failures with ceftriaxone and azithromycin, the first-line agents for gonorrhoea treatment are reported and antimicrobial-resistant *Neisseria gonorrhoeae* is an urgent public health threat. Our aims were to determine antimicrobial resistance rates, resistance determinants and phylogeny of *N. gonorrhoeae* in Ireland, 2014–2016. Overall, 609 isolates from four University Hospitals were tested for susceptibility to extended-spectrum cephalosporins (ESCs) and azithromycin by the MIC Test Strips. Forty-three isolates were whole-genome sequenced based on elevated MICs. The resistance rate to ceftriaxone, cefixime, cefotaxime and azithromycin was 0, 1, 2.1 and 19%, respectively. Seven high-level azithromycin-resistant (HLAzi-R) isolates were identified, all susceptible to ceftriaxone. Mosaic *penA* alleles XXXIV, X and non-mosaic XIII, and G120K plus A121N/D/G (PorB1b), H105Y (MtrR) and A deletion (*mtrR* promoter) mutations, were associated with elevated ESC MICs. A2059G and C2611T mutations in 23S rRNA were associated with HLAzi-R and azithromycin MICs of 4–32 mg/L, respectively. The 43 whole-genome sequenced isolates belonged to 31 NG-MAST STs. All HLAzi-R isolates belonged to MLST ST1580 and some clonal clustering was observed; however, the isolates differed significantly from the published HLAzi-R isolates from the ongoing UK outbreak. There is good correlation between previously described genetic antimicrobial resistance determinants and phenotypic susceptibility categories for ESCs and azithromycin in *N. gonorrhoeae*. This work highlights the advantages and potential of whole-genome sequencing to be applied at scale in the surveillance of antibiotic resistant strains of *N. gonorrhoeae*, both locally and internationally.

Keywords *Neisseria gonorrhoeae* · Ireland · Resistance determinants · MLST · NG-MAST · High-level azithromycin-resistant *Neisseria gonorrhoeae*

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Introduction

Gonorrhoea is one of the most prevalent sexually transmitted infections (STIs) globally, with an estimated 78 million cases of gonorrhoea in 15–49-year-olds in 2012 [1, 2]. The aetiological agent *Neisseria gonorrhoeae* causes both urogenital infections, mostly urethritis and cervicitis, and extragenital infections such as pharyngeal and rectal infections, and may result in severe complications such as ectopic pregnancy, infertility and increased HIV transmission [3, 4].

Gonococci have developed resistance to all first-line antimicrobials recommended for treatment of gonorrhoea since penicillin was introduced in the 1940s [5]. Antimicrobial-resistant *N. gonorrhoeae* is now an urgent public health threat

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globally [1, 5–8]. Increasing resistance to and treatment failures with cefixime in the last decade lead to the introduction of dual therapy, mainly ceftriaxone 250–500 mg and azithromycin 1–2 g, in an attempt to avoid treatment failure, and most importantly to prevent development of resistance and/or spread of resistant strains [1, 8, 9]. However, resistance, including high-level resistance, has been reported to both ceftriaxone and azithromycin. High-level ceftriaxone resistance (MIC ≥ 2 mg/L) has been identified in four sporadic isolates in Japan, France and Spain [10–12]. High-level azithromycin-resistant (HLAzi-R) isolates (MIC ≥ 256 mg/L) have been identified in many countries worldwide, including Ireland [13–18]. Alternative treatment options will undoubtedly be necessary in the near future. Enhanced understanding of the national and international emergence and transmission of antimicrobial-resistant *N. gonorrhoeae* strains is a priority. Whole-genome sequencing (WGS) provides an ideal solution for microepidemiology, macroepidemiology and identification of molecular antimicrobial resistance determinants [19].

The aims of this study were to determine the rates of resistance to extended-spectrum cephalosporins (ESCs) and azithromycin among gonococci in Ireland, to identify antimicrobial resistance determinants and determine the molecular epidemiology using WGS in the most antimicrobial-resistant subset of isolates, including assessing relatedness of HLAzi-R isolates from Ireland and the UK.

Materials and methods

Sample collection and identification

N. gonorrhoeae isolates were collected from four University Hospitals in Ireland (East, South East, West and Midlands), with the majority of isolates (84.4%) originating from Dublin (East). Isolates from all clinical samples which had been prospectively collected at St. James's Hospital (SJH) (2014–2016) and stored on Microbank cryobeads at − 70 °C were recovered for testing. Clinical isolates retrieved from or referred to SJH by other tertiary referral or regional hospitals in Ireland were also examined. A total of 609 isolates were recovered from being frozen: 513 (84.4%) from East, 39 (6.4%) from South East, 31 (5.1%) from West, and 26 (4.3%) from Midlands. Ninety isolates were not viable, and the majority of which were from 2014. The majority of isolates, 329 (53.9%) were collected in 2016, followed by 166 (27.2%) from 2015 and 95 (15.6%) from 2014. Isolates were cultured on chocolate agar prior to confirmation of *N. gonorrhoeae* (Gram-stained microscopy and MALDI-ToF mass spectrometry), antimicrobial susceptibility testing and DNA extraction. Fifteen additional isolates, sequenced previously as part of a different study, were also included in the phylogenetic analysis for this chapter [13, 20].

Antimicrobial susceptibility testing

MICs (mg/L) of ceftriaxone, cefixime, cefotaxime, azithromycin and ertapenem were determined using MIC Test Strips (Liofilchem Roseto degli Abruzzi, Italy), according to manufacturer's instructions. Quality control was performed using the *N. gonorrhoeae* reference strain ATCC 49226. EUCAST clinical breakpoints [21] were used to categorise isolates as resistant (susceptible): ceftriaxone/cefixime/cefotaxime MIC > 0.125 mg/L (MIC ≤ 0.125 mg/L) and azithromycin MIC > 0.5 mg (MIC ≤ 0.25 mg/L). No resistance breakpoints are published by any organisation for ertapenem, but the EUCAST epidemiological cut-off value (ECOFF) is 0.064 mg/L [22].

DNA extraction

Forty-three isolates (7.0%) were selected for WGS based on showing decreased susceptibility or resistance to ESCs and/or resistance or high-level resistance to azithromycin (MIC ≥ 256 mg/L). DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purified DNA was preserved at − 80 °C prior to WGS.

Whole-genome sequencing

Sequencing libraries of *N. gonorrhoeae* genomic DNA were generated using the NexteraXT library preparation kit (Illumina, Eindhoven, the Netherlands), according to manufacturer's instructions, and sequenced on an Illumina MiSeq instrument at the TrinSeq sequencing lab (Trinity College Dublin) using MiSeq v3 reagents. All short-read data obtained in this study has been deposited in the Sequence Read Archive (SRA); project accession number PRJNA473385.

Read mapping and phylogenetic analysis

Delineation of *N. gonorrhoeae* isolate phylogeny was achieved by read mapping to the *N. gonorrhoeae* WHO reference genome WHO N (LT591910.1) and subsequent analysis of core single nucleotide polymorphisms (SNPs) identified using the variant calling tool Snippy (https://github.com/tseemann/snippy). Genome alignments were generated and masked for recombination using Gubbins (https://github.com/sanger-pathogens/gubbins) and a subsequent maximum-likelihood phylogenetic tree was constructed using the generalised time reversible (GTR) substitution model in PhyML [23, 24]. A pairwise comparison table comparing total SNP differences between each isolate pair was generated using a custom python script ('vcf-to-pairwise-distance2.py'). To reproduce the described phylogenetic analysis, a Docker image has been made available at the following link:

https://hub.docker.com/r/nsilico/tree-service/. The code to make this image is available here: https://github.com/blawlor/snippygubbins.

Bioinformatic analysis of antimicrobial resistance determinants

De novo genome assembly was performed using the DTU centre for genomic epidemiology 2.1 assembler pipeline (http://www.genomicepidemiology.org/), which implements the SPAdes genome assembly algorithm [25]. Using genome assembly data, *penA* alleles were assigned a genotype based on the NG-STAR database [26]. The pubMLST tool was employed to determine MLST type and detect antimicrobial resistance determinants (https://pubmlst.org/neisseria/) while additional AMR genes were identified by cross-reference of the ARG-ANNOT database, which contains additional data on AMR (e.g. *ereA*, *ereB*, *ermA*, *ermB*, *ermC*, *mphA*, *mphB*, *mphC*, *mefC*) and plasmid associated genes reported in this study [26, 27]. As there are four copies of the *rrl* (23S rRNA) gene within the *N. gonorrhoeae* genome, reference mapping was performed separately and the number of reads supporting mutations such as the A to G mutations at position 2059 was rounded to the nearest quartile and reported as an estimate of overall allele prevalence within the genome. Finally, a local blast search was performed on de novo assemblies to determine NG-MAST types [28].

Ethics approval

The research proposal was reviewed and approved by the SJH/AMNCH Research and Ethics Committee.

Results

Five-hundred and sixteen isolates (84.6%) originated from STI clinics and only 36 (5.9%) from general practice (GP). The remaining isolates were referred from other hospitals (n = 45), emergency department (n = 1), surgical team (n = 1), ophthalmology (n = 3), and the source was unknown for 7 isolates. Over 90% (n = 553) of isolates were from male patients. The median age was 26 years (mean 28) with a range of 3 to 75 years. Over half the isolates (52.5%, n = 320, of which 35 were female) were from genital sites (urethral/cervical), followed by rectal (26.6%, n = 162, of which 1 was female) and pharyngeal (15.7%, n = 96, of which 5 were female).

No resistance to ceftriaxone was identified, but 1.8% of isolates (n = 11) displayed decreased susceptibility to ceftriaxone (MIC > 0.032 mg/L, CRO-DS), 1% (n = 6) were resistant to cefixime (CFM-R), and 2.1% (n = 13) resistant to cefotaxime (CTX-R). A further 2.5% (n = 15) displayed decreased susceptibility to cefixime (MIC = 0.125 mg/L, CFM-DS) and 7.6% (n = 46) displayed decreased susceptibility to cefotaxime (MIC = 0.125 mg/L, CTX-DS). The MIC_{50} and MIC_{90} of cefotaxime were also higher than corresponding measures for ceftriaxone and cefixime (Table 1).

Forty-four percent of gonococci (n = 268) were non-susceptible to azithromycin (MIC > 0.25 mg/L), including 7 (1.1%) isolates with high-level resistance (MIC > 256 mg/L), 18 (3%) with medium-level resistance (MICs 4–32 mg/L), 88 (14.4%) with low-level resistance (MICs from 1–2 mg/L) and 155 (25.5%) with intermediate susceptibility.

Of the 43 isolates (7%), whole-genome sequenced, 25 belonged to one or more of the following phenotypic groups: CRO-DS (CRO MIC > 0.032 mg/L, n = 9), CFM-R (CFM MIC > 0.125 mg/L, n = 6), CFM-DS (CFM MIC = 0.125 mg/L, n = 14), CTX-R (CTX MIC > 0.125 mg/L, n = 13) and CTX-DS (CTX MIC = 0.125 mg/L, n = 11). The remaining 18 isolates had MICs lower than the above groups but were included based on MICs of azithromycin. The 25 isolates with resistance or decreased susceptibility to ESCs were associated with mosaic *penA* alleles (80%, n = 20) while none (0%) of the 18 isolates showing susceptibility to all ESCs had a mosaic *penA* allele. The majority (n = 6) of the nine CRO-DS isolates carried the mosaic *penA* XXXIV allele (n = 6), while the remainder had mosaic *penA* X (n = 1) or non-mosaic *penA* XIII (n = 2), containing the A501V mutation, alleles. The majority of CFM-DS/R and CTX-R/DS isolates also had these three *penA* allele types: of 14 CFM-DS isolates, 13 were mosaic XXXIV and 1 was non-mosaic XIII; of 6 CFM-R isolates, 5 were mosaic XXXIV and 1 was mosaic X; of 13 CTX-R isolates, 11 were mosaic XXXIV, 1 was mosaic X and 1 non-mosaic XIII; and of 11 CTX-DS isolates, 7 were mosaic XXXIV and 1 was non-mosaic XIII. The 18 isolates with lower ESC MICs contained mainly non-mosaic *penA* II, XIV, XIX, XLIV and XLIX. Additional antimicrobial resistance determinants, NG-MAST STs and MLST STs of all isolates are summarised in Table 2.

All HLAzi-R (MIC > 256 mg/L) isolates had the characteristic A2059G mutation in 2–4 alleles of the 23S rRNA gene, i.e. two mutated alleles in one isolate, three in two isolates and all four alleles mutated in four isolates. All seven HLAzi-R isolates also had an azithromycin resistance mutation in *mtrR*

Table 1 MIC_{50}, MIC_{90} and MIC ranges for five antimicrobials when examining Irish *Neisseria gonorrhoeae* isolates (n = 609) from 2014 to 2016

Antimicrobial	MIC_{50}	MIC_{90}	MIC range
Ceftriaxone	0.008 mg/L	0.016 mg/L	≤ 0.002–0.125 mg/L
Cefixime	0.016 mg/L	0.032 mg/L	≤ 0.016–0.25 mg/L
Cefotaxime	0.032 mg/L	0.064 mg/L	≤ 0.002–0.5 mg/L
Ertapenem	0.016 mg/L	0.032 mg/L	≤ 0.002–0.094 mg/L
Azithromycin	0.025 mg/L	1 mg/L	≤ 0.016– > 256 mg/L

Table 2 Summary of MICs, NG-MAST STs, MLST STs and antimicrobial resistance determinants for the 43 sequenced isolates

Isolate (year)	NG-MAST	MLST	MIC (mg/L)											
			PEN	CIP	AZM	CRO	CFM	TET	CTX	ETP	CN	GEMI	SPC	FOS
006 (2013)	2475	1580	0.25	0.012	> 256	0.016	0.016	1	0.032	0.012	4	0.006	8	16
181798 (2015)	3311	1580	0.25	0.008	> 256	0.008	0.023	0.75	0.047	0.023	4	0.006	8	24
196712 (2016)	649	1580	0.25	0.008	> 256	0.008	0.016	0.75	0.125	0.023	6	0.006	16	12
178487 (2015)	649	1580	0.19	0.008	> 256	0.006	0.023	0.5	0.047	0.032	4	0.004	6	12
193852 (2016)	1443	1580	0.125	≤ 0.002	> 256	0.004	≤ 0.016	0.38	0.006	0.023	4	≤ 0.002	8	8
199856 (2016)	649	1580	0.125	0.004	> 256	0.002	≤ 0.016	0.38	0.004	0.008	3	< 0.002	8	8
191587 (2016)	3311	1580	0.25	0.008	> 256	0.008	0.032	1	0.064	0.016	3	0.004	8	12
67818 (2014)	2400	1587	0.19	> 32	6	0.012	0.023	0.38	0.064	0.012	3	1.5	8	8
190440 (2016)	3750	1588	4	> 32	0.032	0.004	0.016	32	0.032	0.012	4	4	4	12
420132 (2016)	NM	13144	> 32	2	0.064	0.008	≤ 0.016	0.5	0.008	0.012	8	0.5	12	12
71324 (2014)	1582	1588	4	> 32	0.125	0.047	0.19	24	0.19	0.094	0.5	3	6	6
016 (2013)	11645	1901	2	> 32	1	0.047	0.19	1	0.25	0.032	6	1.5	12	32
035 (2016)	NM	1901	0.75	> 32	1	0.047	0.125	0.75	0.125	0.032	4	1.5	6	24
286088 (2014)	3806	1901	0.75	> 32	0.38	0.023	0.094	1	0.19	0.032	6	0.75	16	24
76972 (2014)	3431	1901	1.5	> 32	0.75	0.047	0.19	1	0.25	0.032	4	2	12	32
176561 (2015)	4936	1901	0.75	> 32	0.5	0.016	0.125	0.5	0.19	0.032	6	1.5	8	12
501373 (2016)	5622	1901	0.25	16	0.5	0.032	0.125	1	0.125	0.023	6	2	8	32
024 (2012)	1407	1901	1.5	> 32	0.38	0.094	0.25	1	0.38	0.064	4	1.5	8	48
009 (2013)	1407	1901	1	> 32	0.75	0.064	0.125	1.5	0.125	0.032	4	1.5	12	48
194698 (2016)	1407	1901	0.25	16	0.5	0.016	0.064	0.5	0.19	0.047	4	3	8	48
191262 (2016)	1407	1901	0.5	> 32	0.5	0.023	0.125	1	0.125	0.032	4	1.5	8	24
422671 (2016)	3158	1901	0.5	> 32	0.5	0.064	0.125	2	0.25	0.064	4	2	16	12
179501 (2015)	11690	1901	0.25	3	0.25	0.016	0.094	0.25	0.094	0.023	8	0.75	8	12
422102 (2016)	3158	1901	1	32	1	0.032	0.125	1	0.38	0.032	8	2	16	32
81513 (2015)	2212	1901	0.75	> 32	0.5	0.032	0.19	0.75	0.125	0.064	6	2	12	16
178526 (2015)	8953	1901	1	> 32	1	0.032	0.19	0.75	0.25	0.032	6	1	8	16
68931 (2014)	3806	1901	1	> 32	0.5	0.032	0.125	1	0.25	0.047	8	1.5	12	12
425204 (2016)	NM	7359	0.25	0.016	0.5	0.016	0.125	0.125	0.125	0.032	4	0.006	16	12
193058 (2016)	NM	7363	0.125	8	0.5	0.008	0.016	0.38	0.047	0.016	6	12	6	8
197747 (2016)	14449	7363	0.25	2	16	0.016	0.032	0.5	0.094	0.012	4	2	12	8
425657 (2016)	9184	7363	0.25	16	4	0.016	0.032	1	0.012	0.006	3	1.5	8	16
177574 (2015)	2318	13143	1	8	0.5	0.047	0.094	0.5	0.125	0.016	3	1	8	16
168628 (2016)	2318	7827	1	2	0.25	0.064	0.064	1	0.25	0.012	4	0.38	12	8
422322 (NA)	5624	8143	> 32	2	0.5	0.008	≤ 0.016	0.5	0.016	0.004	8	0.5	16	12
008 (2013)	5624	8143	> 32	3	0.5	0.008	≤ 0.016	0.5	0.023	0.006	6	0.75	12	12
166587 (2016)	12302	9363	0.25	4	8	0.008	0.016	0.75	0.125	0.016	6	2	12	12
180505 (2015)	3935	9363	0.5	0.032	2	0.012	0.023	2	0.064	0.016	16	0.012	12	24
178575 (2015)	2992	9363	0.19	0.004	32	0.003	0.016	0.38	0.012	0.012	6	0.003	6	16
41277 (2014)	2992	9363	0.125	0.004	12	0.003	≤ 0.016	0.25	0.012	0.008	4	0.003	6	12
179411 (2015)	12302	9363	0.5	12	4	0.008	0.023	0.5	0.064	0.016	6	1	12	24
039 (2015)	NM	9903	1.5	4	0.25	0.012	0.016	8	0.016	0.016	6	1.5	8	96
82432 (2015)	7867	13142	0.75	> 32	1	0.023	0.125	1	0.25	0.032	6	6	12	24
86606 (2015)	4244	11428	0.25	0.006	0.5	0.008	0.094	0.38	0.064	0.047	6	0.004	8	24

Table 2 (continued)

Isolate (year)	MIC (mg/L) BL	*penA*	*ponA*	*porB*	TEM	*gyrA*	*parC*	*mtrR*	*mtrR* promoter	*tetM*	*rpld*	23S rRNA
Isolate (year)	MIC (mg/L) BL	*penA*	*ponA*	*porB*	TEM	*gyrA*	*parC*	*mtrR*	*mtrR* promoter	*tetM*	*rpld*	23S rRNA
006 (2013)	–	Non-mosaic type II	WT	A121S	–	WT	WT	G45D	A12C	–	WT	A2059G (4/4)
181798 (2015)	–	Non-mosaic type II	WT	A121S	–	WT	WT	G45D	WT	–	WT	A2059G (3/4)
196712 (2016)	–	Non-mosaic type II	WT	A121S	–	WT	WT	G45D	WT	–	WT	A2059G (4/4)
178487 (2015)	–	Non-mosaic type II	WT	A121S	–	WT	WT	G45D	WT	–	WT	A2059G (3/4)
193852 (2016)	–	Non-mosaic type II	WT	A121S	–	WT	WT	G45D	A13 del	–	WT	A2059G (2/4)
199856 (2016)	–	Non-mosaic type II	WT	A121S	–	WT	WT	G45D	WT	–	WT	A2059G (4/4)
191587 (2016)	–	Non-mosaic type II	WT	A121S	–	WT	WT	WT	WT	–	WT	A2059G (4/4)
67818 (2014)	–	Non-mosaic type 49	L421P	G120K A121D	–	S91F D95G	E91G	WT	A13 del	–	WT	C2611T (4/4)
190440 (2016)	+	Non-mosaic type XIX	L421P	G120K A121G	TEM-198	S91F A92P D95A	E91K S87K	A39T	WT	tetM	WT	WT
420132 (2016)	+	Non-mosaic type II	WT	WT	TEM-75	S91F D95G	E91G	A39T	WT	–	WT	WT
71324 (2014)	+	Mosaic type X	L421P	G120K A121G	TEM-198	S91F D95A	E91K S87K	A39T	WT	tetM	WT	C2611T (1/4)
016 (2013)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
035 (2016)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
286088 (2014)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
76972 (2014)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
176561 (2015)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
501373 (2016)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95A	S87R	WT	A13 del	–	WT	WT
024 (2012)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
009 (2013)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
194698 (2016)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	G71D	WT
191262 (2016)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
422671 (2016)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
179501 (2015)	–	Mosaic XXXIV	L421P	A121S	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
422102 (2016)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
81513 (2015)	–	Mosaic XXXIV	L421P	G120K A121D	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
178526 (2015)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
68931 (2014)	–	Mosaic XXXIV	L421P	G120K A121N	–	S91F D95G	S87R	WT	A13 del	–	WT	WT
425204 (2016)	–	Mosaic XXXIV	WT	WT	–	WT	WT	WT	WT	–	WT	WT
193058 (2016)	–	Non-mosaic type 44	L421P	WT	–	S91F D95G	E91G S87I	WT	A13 del	–	WT	WT
197747 (2016)	–	Non-mosaic type 44	L421P	WT	–	S91F D95G	E91G	WT	A13 del	–	WT	C2611T (4/4)

Table 2 (continued)

Isolate (year)	MIC (mg/L)	*penA*	*ponA*	*porB*	TEM	*gyrA*	*parC*	*mtrR*	*mtrR* promoter	*tetM*	*rpld*	23S rRNA
	BL											
425657 (2016)	–	Non-mosaic type 44	L421P	G120K A121D	–	S91F D95G	E91G	G45D	A13 del	–	WT	C2611T (4/4)
177574 (2015)	–	Non-mosaic type XIII	L421P	G120K A121D	–	S91F D95G	D86N	G45D	A13 del	–	WT	WT
168628 (2016)	–	Non-mosaic type XIII	L421P	G120K A121D	–	S91F D95G	D86N	G45D	A13 del	–	WT	WT
422322 (NA)	+	Non-mosaic type II	WT	G120D A121G	TEM-141	S91F D95A	D86N	A39T	WT	–	WT	WT
008 (2013)	+	Non-mosaic type II	WT	G120D A121G	TEM-141	S91F D95A	D86N	WT	A13 del	–	WT	WT
166587 (2016)	–	Non-mosaic type II	WT	G120K A121N	–	S91F D95A	D86N	WT	A13 del	–	WT	WT
180505 (2015)	–	Non-mosaic type II	WT	G120K A121N	–	WT	WT	WT	WT	–	WT	WT
178575 (2015)	–	Non-mosaic type II	WT	WT	–	WT	WT	A39T	WT	–	WT	C2611T (4/4)
41277 (2014)	–	Non-mosaic type II	WT	WT	–	WT	WT	A39T	WT	–	WT	C2611T (4/4)
179411 (2015)	–	Non-mosaic type II	WT	G120K A121N	–	S91F D95A	D86N	WT	A13 del	–	WT	WT
039 (2015)	+	Non-mosaic type XIV	WT	G120N A121K	TEM-141	S91F D95A	S87N E91Q	WT	WT	tetM	WT	WT
82432 (2015)	–	Mosaic XXXIV	L42IP	G120K A121D	–	S91F D95A	D86N	A39T	A13 del	–	WT	WT
86606 (2015)	–	Mosaic XXXIV	WT	WT	–	WT	WT	A39T	WT	–	WT	WT

(G45D), and one of the isolates also had a single-base pair (A) deletion in the repeated sequence of the *mtrR* promoter. All seven HLAzi-R isolates were assigned as MLST ST1580. Three of the HLAzi-R isolates belonged to NG-MAST ST649, two were of NG-MAST ST3311 and the remaining two were single NG-MAST ST2475 and ST1443 (Table 3).

The remaining isolates formed two groups based on azithromycin MICs, those with azithromycin MICs ranging from 4 to 32 mg/L ($n = 7$) and those with MICs ≤ 2 mg/L ($n = 29$). Five of the seven isolates with medium-level resistance to azithromycin (MIC 4–32 mg/L) had a C2611T mutation in all four alleles of the 23S rRNA gene, and two were wild type (MIC = 4 mg/L and 8 mg/L). These isolates were assigned as MLST ST9363 ($n = 4$), ST 7363 ($n = 2$) and ST1587 ($n = 1$) and NG-MAST ST2992 ($n = 2$), ST12302 ($n = 2$), ST2400 ($n = 1$), ST1449 ($n = 1$) and ST9184 ($n = 1$) (Table 4).

All isolates with azithromycin MICs < 2 mg/L ($n = 29$) had wild-type 23S rRNA, except for one isolate which had a C2611T mutation in one of the four 23S rRNA gene alleles (azithromycin MIC = 0.125 mg/L). Sixteen of these 29 isolates were MLST ST1901 with four of these isolates NG-MAST ST1407. The second most common MLST ST was ST1588 ($n = 3$).

A single-base pair (A) deletion in the repeated sequence of the *mtrR* promoter was present in 21/29 (72%), 4/7 (57%) and 1/7 (14%) of the isolates with azithromycin MICs of 0.032–2 mg/L, 4–32 mg/L and > 256 mg/L, respectively. No *erm*, *ere*, *mef* or *mph* genes were detected.

A phylogenetic tree based on the whole-genome sequence data was constructed. Core SNP sites in 58 whole-genome sequenced strains compared to the NCCP11945 genome were used as the basis for delineating strain phylogeny (Fig. 1).

The phylogeny revealed that HLAzi-R isolates NGSJH11, 181798, 191587 and 178487 belonged to the same MLST ST (ST1580) while differing by < 5 SNPs in their genome sequences (Fig. 1). This suggests potential linked transmission sources or the recent emergence of ST1580 in Ireland, although the latter is unlikely since it has circulated in Ireland since at least 2008 possibly reflecting a very conserved genome [13]. NG-MAST STs in these seven HLAzi-R isolates included ST649 ($n = 3$), ST3311 ($n = 2$), ST2475 ($n = 1$) and ST1443 ($n = 1$). Isolates UHW24 and UH9 belonged to MLST ST1901 and were genetically indistinguishable (0 SNP differences) pointing to a shared source of transmission, while isolates 4226171 and 422102 (also belonging to ST1901) were closely related to each other but differed by 166 SNPs from UHW24 and UH9. The MLST ST1901 was also distinguished by decreased susceptibility to the ESCs. Isolates exhibiting CFM-R ($n = 5$), CFM-DS ($n = 10$) and CRO-DS ($n = 6$) were observed in this clade but no high-level resistance to ESCs or azithromycin was found (Fig. 1). Other MLST STs where potential transmission events were supported included ST1587/ST7363 (isolates 67818 and

Table 3 Summary of NG-MAST, MLST and antimicrobial resistance determinants of seven *Neisseria gonorrhoeae* isolates with high-level resistance to azithromycin (MICs > 256 mg/L)

Isolate (year)	NG-MAST	MLST	AZM MIC (mg/L)	*mtrR*	*mtrR* promoter	23S rRNA	*erm A/B/C/F* *ereA/B* *mefA* *mph A/B/C*
006 (2013)	2475	1580	> 256	G45D	A12C	A2059G (4/4)	–
196712 (2016)	649	1580	> 256	G45D	WT	A2059G (4/4)	–
178487 (2015)	649	1580	> 256	G45D	WT	A2059G (3/4)	–
199856 (2016)	649	1580	> 256	G45D	WT	A2059G (4/4)	–
193852 (2016)	1443	1580	> 256	G45D	A13 del	A2059G (2/4)	–
191587 (2016)	3311	1580	> 256	G45D	WT	A2059G (4/4)	–
181798 (2015)	3311	1580	> 256	G45D	WT	A2059G (3/4)	–

NGSJH12) and ST9363 (isolates: 178575/41277 and 166587/179411) clades, where genetically related isolates were observed while the two identified as ST7827 and ST13143 differed by only 11 SNPs.

The Irish HLAzi-R isolates (all MLST ST1580) identified in the current study were also compared to HLAzi-R MLST ST1580 isolates from an ongoing outbreak in the UK (Sequence Read Archive accession PRJEB14933) to establish potential international spread of HLAzi-R ST1580 strains (Fig. 2). There was significant difference between these isolates (> 100 SNP).

Discussion

Regular and quality-assured surveillance of gonococcal antimicrobial susceptibility is essential for empiric treatment guidelines. In Ireland, ESC resistance is currently not a significant problem since all isolates in this study were susceptible to ceftriaxone, and resistance to cefixime and cefotaxime was relatively rare, i.e. 1 and 2.1%, respectively. Reports from the European Gonococcal Antimicrobial Surveillance Programme (EURO-GASP) have shown that in 2015, 1.7% of isolates were cefixime resistant compared to 2.0% in 2014, while ceftriaxone resistance was detected in only one (0.05%) isolate in 2015, compared with five (0.2%) in 2014 [29, 30].

Azithromycin is part of the empiric first-line dual therapy regimen (along with ceftriaxone) adopted by many countries for treatment of uncomplicated anogenital or pharyngeal gonorrhoea in adults [8]. Although the resistance proportion of 18.6% found in the current study was higher than the majority of reports from elsewhere in Europe, the MIC distribution for azithromycin showed the majority of resistant isolates had MICs of 1–2 mg/L (low-level resistance) and, in general, most isolates had MICs falling in the narrow range of 0.125–0.5 mg/L (73.6%) as previously reported [31].

Table 4 Summary of NG-MAST, MLST and antimicrobial resistance determinants of seven isolates displaying medium-level azithromycin resistance (MICs = 4–32 mg/L)

Isolate (year)	NG-MAST	MLST	AZM MIC (mg/L)	*mtrR*	*mtrR* promoter	23S rRNA	*erm A/B/C/F* *ereA/B* *mefA* *mph A/B/C*
67818 (2014)	2400	1587	6	D79N T86A H105K	A13 del	C2611T (4/4)	–
197747 (2016)	14449	7363	16	Stop codon	A13 del	C2611T (4/4)	–
425657 (2016)	9184	7363	4	D79N T86A H105K	A13 del	C2611T (4/4)	–
178575 (2015)	2992	9363	32	A39T R44H	WT	C2611T (4/4)	–
41277 (2014)	2992	9363	12	A39T R44H	WT	C2611T (4/4)	–
179411 (2015)	12302	9363	4	D79N	A13 del	WT	–
166587 (2016)	12302	9363	8	D79N	A13 del	WT	–

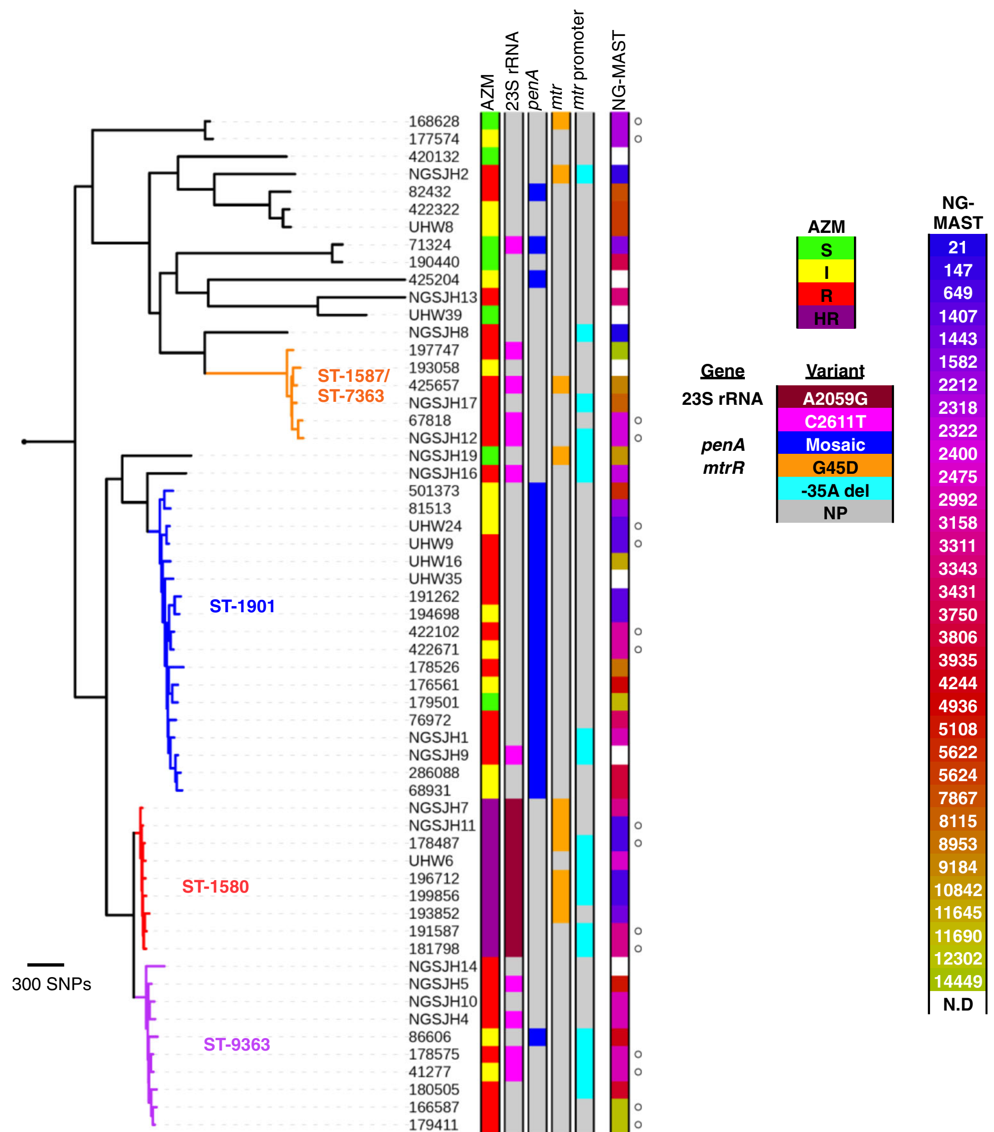

Fig. 1 Phylogenetic analysis based on whole-genome sequences of Irish *N. gonorrhoeae* isolates (PRJNA473385) illustrating resistance to azithromycin (AZM), identified resistance mutations and NG-MAST type. The most prevalent MLST ST groups are identified by colour coding along with the AZM resistance level (S; ≤ 0.25 mg/L, I; > 0.25–≤ 0.5 mg/L, R; > 0.5 mg/L, HR; ≥ 256 mg/L). The presence of detected mutations in the 23S rRNA gene (A2059G and C2611T) and mosaic *penA* alleles and mutations in the *mtrR* coding (G45D) and promoter regions (− 35A del) are indicated by coloured strips according to the key to the right of the figure (NP, 'not present'). Identified NG-MAST ST types are also colour-indicated (ND, 'not determined'). Highly related strains (< 20 SNPs) are highlighted by open circles. Genome sequences from 15 additional Irish *N. gonorrhoeae* isolates, which can be accessed in the Sequence Read Archive (accession PRJNA275092), were included in the tree

Of the azithromycin-resistant isolates found in this study, a small proportion ($n = 7$, 1.1%) displayed high-level resistance to azithromycin (MIC > 256 mg/L). However, this is a higher proportion than reported in other countries or regions that frequently examined larger numbers of isolates, such as 0 and 18% in China ($n = 0/485$, 2009–2013 and $n = 21/118$, 2011–2012, respectively), 0.002% in the USA ($n = 1/44{,}144$, 2005–2013), and 0.04–0.2% in Europe ($n = 2/1902$, $n = 1/1994$, $n = 1/2151$ and $n = 5/2134$ in 2011, 2013, 2014 and 2015, respectively) and 0.7% in Canada ($n = 2/2800$, 2010–2013) [29–37].

In general, the recorded resistance rates to azithromycin vary somewhat from country to country across the world which is due to differences in true resistance rates but may also reflect application of different resistance breakpoints and that many circulating gonococcal strains have MICs close to the resistance breakpoint. In this study, the proportion of isolates resistant to azithromycin was higher than older reports from elsewhere in Europe, including Amsterdam (1.2%; 2012–2015) and South-West Germany (7.1%; 2010–2015) [31, 38], but was lower or similar to other countries such as Switzerland (23.6%; 2009–2012) and Hungary (15.9%; 2013) [39, 40]. The rate of azithromycin resistance found in the 2015 EURO-GASP surveillance is similar to the rates found in South-West Germany, at 7.1% [30].

The major resistance determinant at which mutations are known to decrease susceptibility to ESC MICs is the *penA* gene and particularly the presence of a mosaic *penA* gene. Two mosaic *penA* allele types were identified in this study among the CRO-DS isolates and both these mosaic *penA* alleles are associated with decreased susceptibility to ESCs and are prevalent worldwide [19, 41–45]. Most ($n = 6$) of the nine CRO-DS isolates had the mosaic *penA* XXXIV allele. The mosaic *penA* XXXIV has been shown to be associated with increased ESC MICs [19, 41]. This is also the *penA* allele that

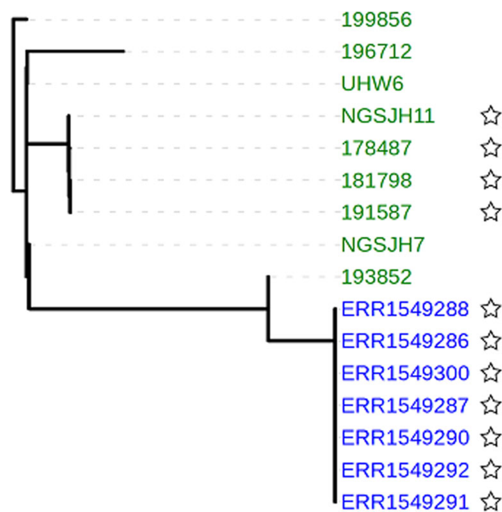

Fig. 2 Whole-genome sequence comparison of Irish and UK high-level azithromycin-resistant (HLAzi-R) ST1580 isolates. Irish and UK strains are indicated in green and blue font respectively. Stars indicate putative transmission events within specific clades based on observed SNP differences of < 5 among isolates. While geographically contained clonal clusters are observed, a single Irish isolate (193852) showed some similarity with the UK outbreak strains, but differed significantly (> 100 SNPs)

has been associated with the majority of verified ESC treatment failures [46–51]. Of the three remaining CRO-DS isolates, two had the non-mosaic *penA* XIII (A501V) allele and one had a mosaic *penA* X allele. The mosaic *penA* X allele has also been associated with ESC resistance and treatment failures [19, 45, 52]. In 1995–2005 in Japan, 129/149 CFM-R gonococcal isolates were shown to possess the mosaic *penA* X alleles [45]. Non-mosaic *penA* XIII (containing the A501V and P551S resistance mutations) was the most common allele type in 56/95 isolates from Korea (2011–2013), which included 21/25 of the ESC-resistant isolates [42].

Other mutations found to be associated with elevated ESC MICs in this study included the additional ESC resistance mutations G120K plus A121N/D/G alterations in PorB1b, and an A deletion in the inverted repeated sequence of the *mtrR* promoter. These mutations have been associated with increased MICs to ESCs [39, 53–55].

N. gonorrhoeae HLAzi-R is specifically associated with an A2059G mutation in the peptidyltransferase loop in domain V in the 23S rRNA, when it is present in 3 or 4 alleles and this has been reported worldwide [13, 14, 16–18, 35, 56, 57]. As described in this study, seven of the 43 isolates sequenced were HLAzi-R, one of which had an A2059G mutation in only 2 out of the 4 23S rRNA gene alleles, the remaining isolates had 3/4 ($n = 2$) or 4/4 ($n = 4$) mutated alleles. HLAzi-R gonococcal isolates with only 2 alleles containing the A2059G mutation have rarely been reported [58]. This isolate also had a G45D alteration in *mtrR* that all the seven HLAzi-R isolates had; however, it was the only HLAzi-R isolate which also had an A deletion in the inverted repeat sequence of the *mtrR* promoter. Of 25 internationally reported HLAzi-R isolates, at least six had a G45D substitution in *mtrR* and at least nine had an A deletion in the promoter region [13, 14, 16–18, 35, 56, 57, 59].

The 23S rRNA mutation C2611T found in the medium-level azithromycin-resistant isolates in the current study has been widely reported to be associated with resistance to azithromycin, MICs ranging from 1 to 64 mg/L with the number of mutated alleles ranging from 1 to 4, but mostly three copies have been associated with azithromycin MICs ≥ 4 mg/L [13, 19, 35, 37, 57, 60–62]. One azithromycin-susceptible isolate in the current study (AZM MIC = 0.125 mg/L) had one C2611T mutated 23S rRNA allele, but there were other reports of gonococcal isolates with one C2611T mutated allele, associated with AZM MICs of 0.38, 1 and 2 mg/L [25, 62]. Grad et al. reported a positive predictive value of 99% for decreased susceptibility to azithromycin if there were ≥ 2 C2611T mutated loci (C2611T), and interestingly, having one C2611T mutated loci had the same negative predictive value for decreased susceptibility to azithromycin as wild-type 23S rRNA [63].

Given that A2059G (in ≥ 3 of the 4 alleles) and C2611T (in ≥ 3 of the 4 alleles) mutations in the 23S rRNA gene are only found in gonococcal isolates with AZM MICs ≥ 4 mg/L, absence of these mutations may provide a means of assessing whether azithromycin may be relied upon for treatment of gonorrhoea if molecular methods are employed rather than phenotypic susceptibility testing.

In the current study, the A deletion in the *mtrR* promoter was present more often in gonococci with lower AZM MICs than in those with higher MICs. In other studies, the presence of this *mtrR* mutation region has been similar across all levels of susceptibility to azithromycin [19, 37, 57]. No *erm*, *ere*, *mef* or *mph* genes were detected in the current study, which is in accordance with the majority of studies investigating azithromycin resistance in *N. gonorrhoeae*.

The genomic analysis confirmed the most prevalent *N. gonorrhoeae* strain types in our study. These included the MLST ST1580, ST9396 and ST1901 lineages as well as the genetic diversity within these genetic clades. Three novel MLST STs were identified among the isolates tested (ST13142, ST13143 and ST13144). Jacobsson et al. reported NG-MAST ST2992 and MLST ST1901 to be the predominant STs out of 75 azithromycin-resistant (MIC > 2 mg/L) isolates from 17 EURO-GASP countries (2009–2014) [57], but in another genomic study examining a larger number of consecutive European isolates from 20 countries in 2013 (n = 1054), NG-MAST ST1407 and MLST ST1901 were the predominant STs [19]. While significant genetic diversity was seen, a number of closely related isolates were observed suggesting putative transmission or shared environmental sources, or, alternatively, the more recent emergence of these strains in Ireland. This highlights the potential of whole-

genome sequencing-based approaches to track disease transmission at a finer and more accurate level.

While this was not the primary focus of the current study, larger and more systematic application of whole-genome sequencing in Ireland could elucidate the emergence and spread of *N. gonorrhoeae* strains at a national level. In terms of international surveillance, the portability of whole-genome sequencing data and possibility for international comparison of whole-genome sequencing data are also noteworthy [19]. In the current study, we exploited this in the comparison of our HLAzi-R MLST ST1580 isolates with those from an ongoing outbreak in the UK. This highlighted the significant differences identified between the HLAzi-R MLST ST1580 isolates in the current study and those of the UK outbreak. Thus, genomic data can be integrated at scale to provide rapid comparisons of disparate databases toward greater international surveillance of gonorrhoea. A more recent UK study reported a sustained transmission of a clonal outbreak of HLAzi-R *N. gonorrhoeae* over several years, the majority of isolates belonging to ST9768 which they stated may be a descendant of ST649 [58]. This study also found that HLAzi-R may emerge quite quickly from azithromycin-susceptible or low-level azithromycin-resistant strains, making WGS essential for identifying transmission events [58].

The limitations of the current study included that the isolates were not representative of all gonococcal isolates cultured in Ireland during the study time period (2014–2016), as it was not possible to obtain isolates from all laboratories in Ireland and some frozen isolates were not retrievable ($n = 90$). Similarly, because other tertiary hospitals referred isolates to SJH, this may have led to an overrepresentation of azithromycin resistance. Furthermore, epidemiological data were mainly lacking and no information regarding sexual orientation of the patient, mode of transmission or response to antimicrobial treatment was available. Finally, the study was limited by the small number of ESC-resistant gonococcal isolates available and the small number of isolates that were whole-genome sequenced.

This is the largest antimicrobial resistance study, linked to whole-genome sequencing, of Irish *N. gonorrhoeae* isolates performed to date and highlights the importance of continued antimicrobial susceptibility surveillance and important advantages and potential of whole-genome sequencing to be applied at scale in the surveillance of antimicrobial-resistant strains of *N. gonorrhoeae*, both locally and internationally.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Ethics approval was granted by the Tallaght University Hospital/St. James's Hospital Joint Research Ethics Committee.

Informed consent Not applicable.

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