J Antimicrob Chemother 2016; **71**: 3096–3108
doi:10.1093/jac/dkw288 Advance Access publication 17 July 2016

Journal of Antimicrobial Chemotherapy

The novel 2016 WHO *Neisseria gonorrhoeae* reference strains for global quality assurance of laboratory investigations: phenotypic, genetic and reference genome characterization

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Received 15 March 2016; returned 21 April 2016; revised 2 June 2016; accepted 11 June 2016

Objectives: Gonorrhoea and MDR *Neisseria gonorrhoeae* remain public health concerns globally. Enhanced, quality-assured, gonococcal antimicrobial resistance (AMR) surveillance is essential worldwide. The WHO global Gonococcal Antimicrobial Surveillance Programme (GASP) was relaunched in 2009. We describe the phenotypic, genetic and reference genome characteristics of the 2016 WHO gonococcal reference strains intended for quality assurance in the WHO global GASP, other GASPs, diagnostics and research worldwide.

Methods: The 2016 WHO reference strains ($n=14$) constitute the eight 2008 WHO reference strains and six novel strains. The novel strains represent low-level to high-level cephalosporin resistance, high-level azithromycin resistance and a *porA* mutant. All strains were comprehensively characterized for antibiogram ($n=23$), serovar, prolyliminopeptidase, plasmid types, molecular AMR determinants, *N. gonorrhoeae* multiantigen sequence typing STs and MLST STs. Complete reference genomes were produced using single-molecule PacBio sequencing.

Results: The reference strains represented all available phenotypes, susceptible and resistant, to antimicrobials previously and currently used or considered for future use in gonorrhoea treatment. All corresponding resistance genotypes and molecular epidemiological types were described. Fully characterized, annotated and finished references genomes ($n=14$) were presented.

Conclusions: The 2016 WHO gonococcal reference strains are intended for internal and external quality assurance and quality control in laboratory investigations, particularly in the WHO global GASP and other GASPs, but also in phenotypic (e.g. culture, species determination) and molecular diagnostics, molecular AMR detection, molecular epidemiology and as fully characterized, annotated and finished reference genomes in WGS analysis, transcriptomics, proteomics and other molecular technologies and data analysis.

Introduction

Gonorrhoea is a public health concern globally.[1,2] The impact of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* (gonococci) on the treatment and control of gonorrhoea is a longstanding concern. In the last decade, while retaining AMR to previously used therapeutic drugs, gonococci have developed resistance, including high-level resistance, to the last option for empirical antimicrobial monotherapy, the extended-spectrum cephalosporin (ESC) ceftriaxone.[3–10] Rare failures to treat pharyngeal gonorrhoea with ceftriaxone were verified in several countries.[3,5,11–14]

In response, the WHO,[2] CDC[15] and ECDC[16] have published global and regional action plans to control the transmission and impact of MDR and XDR gonorrhoea. A key component of these

plans is to enhance the surveillance of gonococcal AMR locally, nationally and internationally. The WHO global Gonococcal Antimicrobial Surveillance Programme (GASP) was relaunched in 2009.[2,17] The WHO GASP works in liaison with other GASPs. For example, Euro-GASP is acting in the European Union and European Economic Area[18,19] and national GASPs are active in the USA[20] (http://www.cdc.gov/std/gisp), UK[21] (http://www.hpa.org.uk/Publications/InfectiousDiseases/HIVAndSTIs/GRASPReports/) and many additional countries. This enhanced gonococcal AMR surveillance should monitor trends in resistance, identify newly emerging AMR and inform treatment guidelines in a timely fashion. However, reliable, quality assured and nationally and internationally comparable AMR data are essential. In the absence of uniform AMR testing methodology globally (method parameters, agar media and resistance breakpoints), inter-laboratory comparisons of AMR data are enabled through the use of international reference strains.[22,23] The WHO Collaborating Centre in Örebro, Sweden and the WHO Collaborating Centre in Sydney, Australia have undertaken continuing assessments over many years to select, evaluate and nominate suitable gonococcal strains for use in the internal quality control and external quality assurance of national, WHO regional and international GASPs and for other purposes. In 2009, the 2008 WHO gonococcal reference strains were published.[23] The characterizations of these reference strains ($n=8$) were substantially expanded in the present study. Furthermore, due to the emergence of ESC resistance and high-level azithromycin resistance, five additional strains (with low-level to high-level resistance to ESCs, including resistance associated with ESC treatment failures, and azithromycin) have now been added to the WHO reference strain panel. In recent years, gonococcal *porA* mutants containing *N. meningitidis porA* gene sequences that result in false-negative results in *porA*-based gonococcal nucleic acid amplification tests (NAATs) have been described in several countries.[24,25] One such gonococcal *porA* mutant has also been included among the 2016 WHO gonococcal reference strains ($n=14$).

This study characterized the 2016 WHO *N. gonorrhoeae* reference strains phenotypically [antibiograms, serovars and prolyliminopeptidase (PIP) production] and genetically [AMR plasmid types, molecular resistance determinants, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) STs and MLST STs] and presents finished, fully characterized and annotated reference genomes, which will be exceedingly valuable for quality assurance of WGS, transcriptomics, proteomics and other molecular technologies and data analysis. The 2016 WHO gonococcal reference strains are intended for internal and external quality assurance and quality control in all types of laboratory examinations, particularly in the AMR testing (phenotypic and genetic) in GASPs, as recommended for the WHO global GASP, but also for phenotypic (e.g. culture) and genetic (e.g. NAATs) diagnostics, species determination, genetic AMR detection, molecular epidemiology and genomics.

Materials and methods

Bacterial strains

The 2016 WHO gonococcal reference strains include the previously published 2008 WHO gonococcal reference strains (WHO F, G, K, L, M, N, O and P)[23] and six additional, strictly selected gonococcal strains. The new strains were designated as WHO U (Sweden, 2011),[25] WHO V (Sweden, 2012),[26] WHO W (Hong Kong, 2007),[27] WHO X (H041; Japan, 2009),[5] WHO Y (F89; France, 2010)[7] and WHO Z (A8806; Australia, 2013).[8] All strains were cultivated and preserved as described.[28] All the 2016 WHO gonococcal reference strains ($n=14$) are available at the National Collection of Type Cultures (NCTC; www.phe-culturecollections.org.uk) under the NCTC numbers 13477–13484, 13817–13822 (Table 1).

Serogroup and serovar determination

Serogroup and serovar determination using PhadeBact GC Monoclonal Serovar Test (Bactus AB, Stockholm, Sweden) were performed as described.[29]

Detection of PIP

PIP production was detected using API NH (bioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions.

Antimicrobial susceptibility testing

MICs of 20 antimicrobials were determined using the Etest method (bioMérieux, Solna, Sweden), according to the manufacturer's instructions, on GCRAP agar plates [3.6% Difco GC Medium Base agar (BD Diagnostics, Sparks, MD, USA) supplemented with 1% haemoglobin (BD) and 1% IsoVitalex (BD)]. MICs of solithromycin,[4,14] zoliflodacin (also known as ETX0914 and AZD0914)[4,14] and thiamphenicol were determined using the agar dilution method as previously described. Where breakpoints were available, the susceptible (S), intermediate susceptibility (I) and resistance (R) categorization was based on the interpretative criteria from EUCAST (www.eucast.org). For the antimicrobials where EUCAST does not state any breakpoints, only the consensus MIC values are presented (Table 1). For all strains and antimicrobials, each determination was performed at least three times using new bacterial suspensions on separate batches of agar plates and the consensus MIC was reported. β-Lactamase production was detected using nitrocefin solution (Oxoid, Basingstoke, UK).

Isolation of bacterial DNA

Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Purified DNA was stored at 4°C prior to genetic analysis.

Conventional sequencing

The PCRs, purification of PCR amplicons and conventional sequencing of genes associated with diagnostics, AMR and molecular epidemiology were performed as described.[23]

Sequence alignments were performed using the BioEdit Sequence Alignment Editor (v. 7.0.9.0) software (Ibis Biosciences, Carlsbad, CA, USA). Determinations of NG-MAST STs and MLST STs were performed as described previously.[23,29,30] All genes and determinants (Table 2) were also identified *in silico* from the WGS data.

Genome sequencing

Single-molecule, real-time (SMRT) DNA genome sequencing was performed using the PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) with P5-C3 chemistry, resulting in average sub-read lengths of 28476 bp. One or two SMRT cells were used for each strain to provide high coverage levels for each (average of 165×) (Table S1, available as Supplementary data at *JAC* Online).

De novo assembly of the genomes was conducted using the hierarchical genome assembly process (HGAP3, SMRTAnalysis 2.3.0) workflow. Full chromosome sequences were circularized using Circlator[31] and then

further corrected using Quiver.[32] WHO F and WHO K were manually circularized and corrected with Quiver.[32]

Because large fragments are selected during PacBio library preparation and the longest reads are selected for assembly by the HGAP pipeline, not all plasmids were retrieved in the HGAP assemblies. The cryptic plasmid was not retrieved in six strains (WHO N, O, P, U, V and Z) and the Asian pBlaTEM plasmid in WHO N. Accordingly, Illumina sequencing was also performed on the 14 strains using the HiSeq 2000 2×100 bp platform. Reads were assembled using a pipeline (https://github.com/sanger-pathogens/vr-codebase) developed at the Wellcome Trust Sanger Institute.[33] The missing plasmids were detected as single contigs directly from the Illumina assemblies using ACT.[34] They were circularized manually using the other strains as references.

As a second check, the three types of plasmid detected (pCryptic, pBlaTEM and pConjugative) were compared with their corresponding references in public databases (pJD1, pJD4 and pEP5289, respectively) by mapping all PacBio filtered sub-reads against the reference using BWA MEM.[35] Mapped reads were extracted from the bam files and assembled independently using minimap and miniasm,[36] and the resulting unitigs corrected using Quiver.[32] The initial, Illumina, assemblies of the WHO N and WHO V Asian pBlaTEM plasmids were found to be missing a 1.8 kb region compared with the resulting assemblies from miniasm and the Asian reference plasmid. Thus, miniasm corrected assemblies were used for subsequent steps in these two cases.

Full chromosomes and plasmids were annotated using PROKKA v. 1.11[37] followed by manual curation using Artemis.[38] Additional proteins annotated in *N. gonorrhoeae* FA1090 but not identified in the automatic annotation were checked manually and added when appropriate. InterProScan 5[39] was used to improve annotation of hypothetical proteins. Sequence data for each strain has been deposited in the ENA under BioProject accession no. PRJEB14020.

Genomes were compared through BLAST[40] analyses and visualized using BRIG v. 0.95.[41] The core genome of the 14 references was obtained using Roary.[42] Pairwise core SNPs were calculated and a minimum evolution tree computed using MEGA v6.[43]

Results

Phenotypic characterization

Three (21%) and 11 (79%) of the strains were assigned as serogroup PorB1a (WI) and PorB1b (WII/III), respectively, and in total eight serovars were represented (Table 1). Three (21%) of the strains (WHO G, N and V) were PIP-negative. Four (29%) of the strains (WHO M, N, O and V) produced β-lactamase. The consensus MIC and, where feasible, SIR categorization for each antimicrobial and strain, and the range of MICs for all strains, as determined by the single method used, are also presented. The strains represented all important susceptible, intermediate susceptible and resistant phenotypes and the ranges of resistances seen for most antimicrobials previously or currently recommended in different guidelines, used in gonorrhoea treatment globally or considered for future use. These included strains with high-level resistance to penicillin G, ampicillin, temocillin, ceftriaxone, cefixime, cefuroxime, azithromycin, erythromycin, ciprofloxacin, moxifloxacin, gemifloxacin, tetracycline, spectinomycin, sulfamethoxazole and rifampicin (Table 1).

Genetic characterization

One of the strains (WHO F) contained a WT *penA* allele, five strains (WHO K, W, X, Y and Z) a mosaic *penA* allele (main ESC resistance determinant),[3,4,14] and eight strains displayed the Asp345a alteration in the β-lactam main target penicillin-binding protein 2 (PBP2), which is observed in chromosomally mediated penicillin resistance (Table 2).[3,4] WHO L and WHO Y contained a PBP2 A501V and A501P alteration, respectively, which also increase the MICs of β-lactam antimicrobials including ESCs.[3,4,6,7] Other *penA* mutations that increase the ESC MICs are also presented. Four strains (WHO F, L, N and U) contained a WT *mtrR* promoter region sequence. The remaining strains displayed a deletion of a single nucleotide (A; $n=8$) or an A→C substitution ($n=2$) in the 13 bp inverted repeat of the *mtrR* promoter sequence, resulting in an increased MtrCDE efflux of substrate antimicrobials, e.g. macrolides and β-lactam antimicrobials.[3,4,23] Also WHO L and WHO N had an over-expressed MtrCDE efflux pump due to the mtr_{120} mutation and a deletion of a single nucleotide in *mtrR*, respectively. These mutations result in an additional promoter for *mtrCDE* and a frame-shift, premature stop codon and truncated MtrR, respectively.[23,44] Concerning the *penB* AMR determinant, among the PorB1b strains ($n=11$) 10 displayed mutations in A102 [A102D ($n=9$) and A102N ($n=1$)] and nine additionally a G101K alteration, which mediate decreased permeability of target antimicrobials through the porin PorB1b.[3,4] Twelve strains carried the *ponA* mutation (*ponA1* allele) resulting in the L421P alteration in the second β-lactam target PBP1, which is observed in high-level chromosomally mediated penicillin resistance.[4] Of the β-lactamase-producing strains ($n=4$), two (WHO M and O) carried African-type plasmid and two (WHO N and V) Asian-type plasmid, which all contained bla_{TEM-1} resulting in high-level penicillin resistance.[45,46] In regards of fluoroquinolone resistance, one strain (WHO G) displayed only an S91F mutation in GyrA, subunit A of DNA gyrase (ciprofloxacin MIC = 0.125 mg/L), one (WHO M) a GyrA S91F mutation and a GyrA D95G mutation (ciprofloxacin MIC = 2 mg/L) and the remaining eight ciprofloxacin-resistant strains contained a GyrA S91F mutation, a GyrA D95G/N mutation and one or two amino acid alterations in codons 86–88 of ParC, subunit C of DNA topoisomerase IV (ciprofloxacin MIC = 4–>32 mg/L).[4,47] One strain (WHO O) had a C1192T spectinomycin target mutation in all four alleles of the 16S rRNA gene (spectinomycin MIC >1024 mg/L[48]). WHO U and WHO V possessed the C2611T mutation and A2059G mutation, respectively, in all four alleles of the 23S rRNA gene, which are target mutations causing low-level and high-level resistance to azithromycin.[4,26,49] No azithromycin resistance mutations were found in the *rplD* or *rplV* gene (encoding ribosomal protein L4 and L22, respectively) and none of the macrolide resistance-associated genes *mefA/E* (encoding Mef efflux pump),[50] *ereA* and *ereB* (encoding erythromycin esterase) or *ermA-C* and *ermF* (encoding RNA methylases that block macrolides from binding to the 23S subunit target)[51] were identified in any of the strains. Three of the strains (WHO M, N and P) contained an H552N target mutation in RpoB (encoding RNA polymerase subunit B), resulting in high-level rifampicin resistance.[52] The *tet(M)*-carrying conjugative plasmids, resulting in high-level tetracycline resistance, identified in WHO G and N were of the Dutch plasmid type.[53] All strains except WHO F had the V57M mutation in *rpsJ*, encoding ribosomal protein S10 and involved in chromosomally mediated tetracycline resistance.[54] All strains except WHO F and WHO L contained the R228S mutation in the sulphonamide target dihydropteroate synthase (DHPS), encoded by *folP*, associated with sulphonamide resistance.[55] Finally, the promoter sequence for the *macAB* operon (encoding the MacA-MacB efflux pump) contained the −10 hexamer sequence TAGAAT in all strains. This sequence is

Table 1. Phenotypic characteristics of epidemiological and diagnostic relevance and antimicrobial susceptibility/resistance phenotypes displayed by the 2016 WHO *N. gonorrhoeae* reference strains ($n=14$)

Characteristic	WHO F[a]	WHO G[a]	WHO K[a]	WHO L[a]	WHO M[a]	WHO N[a]	WHO O[a]	WHO P[a]	WHO U	WHO V	WHO W	WHO X	WHO Y	WHO Z
NCTC number	13477	13478	13479	13480	13481	13482	13483	13484	13817	13818	13819	13820	13821	13822
Serogroup	PorB1a	PorB1a	PorB1b	PorB1b	PorB1b	PorB1a	PorB1b	PorB1b	PorB1b	PorB1b	PorB1b	PorB1b	PorB1b	PorB1b
Serovar	Arst	Arst	Bpyust	Brpyust	Bpyust	Arst	Boys	Bopt	Bryust	Bropys	Bpyust	Bpyust	Bpyut	Bpyust
PIP production[b]	Pos	—	Pos	Pos	Pos	—	Pos	Pos	Pos	—	Pos	Pos	Pos	Pos
β-Lactamase (PPNG)[c]	—	—	—	—	Pos	Pos	Pos	—	—	Pos	—	—	—	—
Penicillin G (0.032–>32)[d]	S (0.032)	I (0.5)	CMRNG (2)	CMRNG (2)	PPNG[c] (≥32)	PPNG[c] (>32)	PPNG[c] (>32)	I (0.25)	I (0.125)	PPNG[c] (>32)	CMRNG (4)	CMRNG (4)	I (1)	CMRNG (2)
Ampicillin (0.032–>256)[d,e]	0.032	0.25	2	2	PPNG[c] (8)	PPNG[c] (4)	PPNG[c] (24)	0.064	0.125	PPNG[c] (>256)	2	2	0.5	2
Temocillin (0.064–32)[d,e]	0.064	1	16	4	1	1	4	1	0.5	4	8	32	8	8
Cefuroxime (0.032–16)[d,e]	0.064	0.5	16	8	0.5	0.25	1	0.125	0.032	1	8	8	8	8
Cefixime (<0.016–4)[d]	S (<0.016)	S (<0.016)	LLR (0.25)	S (0.125)	S (<0.016)	S (<0.016)	S (0.016)	S (<0.016)	S (<0.016)	S (<0.016)	LLR (0.25)	HLR (4)	HLR (2)	HLR (2)
Ceftriaxone (<0.002–2)[d]	S (<0.002)	S (0.008)	S (0.064)	LLR (0.25)	S (0.016)	S (0.004)	S (0.032)	S (0.004)	S (0.002)	S (0.064)	S (0.064)	HLR (2)	HLR (1)	LLR (0.5)
Ertapenem (0.004–0.125)[d,e]	0.004	0.008	0.125	0.032	0.016	0.008	0.032	0.008	0.004	0.012	0.064	0.064	0.008	0.032
Erythromycin (0.5–>256)[d,e]	0.5	1	1	2	1	0.5	1	4	>256	>256	2	2	2	4
Azithromycin (0.125–>256)[d]	S (0.125)	S (0.25)	S (0.25)	I (0.5)	S (0.25)	S (0.25)	S (0.25)	R (4)	R (4)	HLR (>256)	I (0.5)	I (0.5)	R (1)	R (1)
Ciprofloxacin (0.004–>32)[d]	S (0.004)	LLR (0.125)	HLR (>32)	HLR (>32)	R (2)	R (4)	S (0.008)	S (0.004)	S (0.004)	HLR (>32)	HLR (>32)	HLR (>32)	HLR (>32)	HLR (>32)
Gemifloxacin (0.004–16)[d,e]	0.004	0.125	16	8	0.5	1	0.008	0.016	0.008	4	16	16	2	8
Moxifloxacin (0.004–16)[d,e]	0.004	0.064	8	16	1	1	0.016	0.032	0.008	8	8	8	4	8
Spectinomycin (8–>1024)[d]	S (16)	S (16)	S (16)	S (16)	S (16)	S (16)	HLR (>1024)	S (8)	S (8)	S (16)	S (16)	S (16)	S (16)	S (16)
Gentamicin (4–8)[d,e]	4	4	4	4	4	4	4	4	4	8	4	4	8	4
Kanamycin (8–32)[d,e]	16	16	16	32	16	16	16	8	8	16	16	16	16	8
Tetracycline (0.25–32)[d]	S (0.25)	TRNG (32)	R (2)	R (2)	R (2)	TRNG (16)	R (2)	I (1)	I (1)	R (4)	R (4)	R (2)	R (4)	R (4)
Chloramphenicol (0.5–8)[d,e]	0.5	2	4	8	4	4	4	4	4	8	8	8	4	8
Thiamphenicol (0.25–4)[d,e]	0.25	0.5	2	4	4	1	2	1	2	4	4	4	4	4
Fosfomycin (8–32)[d,e]	32	32	16	8	32	16	32	32	32	16	16	16	16	16
Rifampicin (0.125–>32)[d,e]	0.125	0.5	0.5	0.5	>32	>32	0.25	>32	0.25	0.5	0.25	0.5	0.5	0.5
Sulfamethoxazole (16–>1024)[d,e]	64	512	128	16	128	256	128	64	32	>1024	64	128	64	128
Solithromycin (0.064–32)[d,e]	0.064	0.064	0.064	0.125	0.064	0.064	0.125	0.5	0.25	32	0.064	0.064	0.125	0.125
Zoliflodacin (0.032–0.125)[d,e]	0.032	0.064	0.064	0.064	0.064	0.125	0.064	0.125	0.064	0.064	0.064	0.064	0.064	0.064

NCTC, National Collection of Type Cultures; S, susceptible; I, intermediate susceptible; R, resistant; LLR, low-level resistant; HLR, high-level resistant; CMRNG, chromosomally mediated resistant *N. gonorrhoeae*; TRNG, plasmid-mediated high-level tetracycline-resistant *N. gonorrhoeae*.

[a]Includes some previously published results.[23] However, additional antimicrobials have been examined and some consensus MICs have slightly changed when additional MIC determinations using different MIC-determining methodologies have been performed.

[b]PIP-negative *N. gonorrhoeae* strains do not produce the enzyme PIP, which can result in doubtful and/or false-negative species identification of *N. gonorrhoeae* using a biochemical or enzyme-substrate test. Global transmission of PIP-negative *N. gonorrhoeae* strains has been identified.[68]

[c]PPNG, penicillinase-producing *N. gonorrhoeae* (always considered resistant to all penicillins independent of identified MIC value, which might slightly vary).

[d]Resistance phenotypes based on MIC (mg/L) using Etest or agar dilution and, where feasible, susceptibility/resistance breakpoints stated by EUCAST (www.eucast.org). The reported MIC values are mean MICs (rounded to whole MIC dilution) and the acceptable range of the MICs for each antimicrobial and the different strains is±one MIC doubling dilution, i.e. when the strains are used in quality control, for example. Note: the consensus MICs shown should be used and interpreted with caution because these were derived using one method only and, consequently, may slightly differ using other methods. However, the identified resistance phenotypes (SIR categorization) should ideally be consistent between different methods.[23,59]

[e]No susceptibility/resistance breakpoints stated by EUCAST (www.eucast.org).

Table 2. Genetic characteristics of relevance for epidemiology, diagnostics and antimicrobial resistance in the 2016 WHO *N. gonorrhoeae* reference strains ($n=14$)

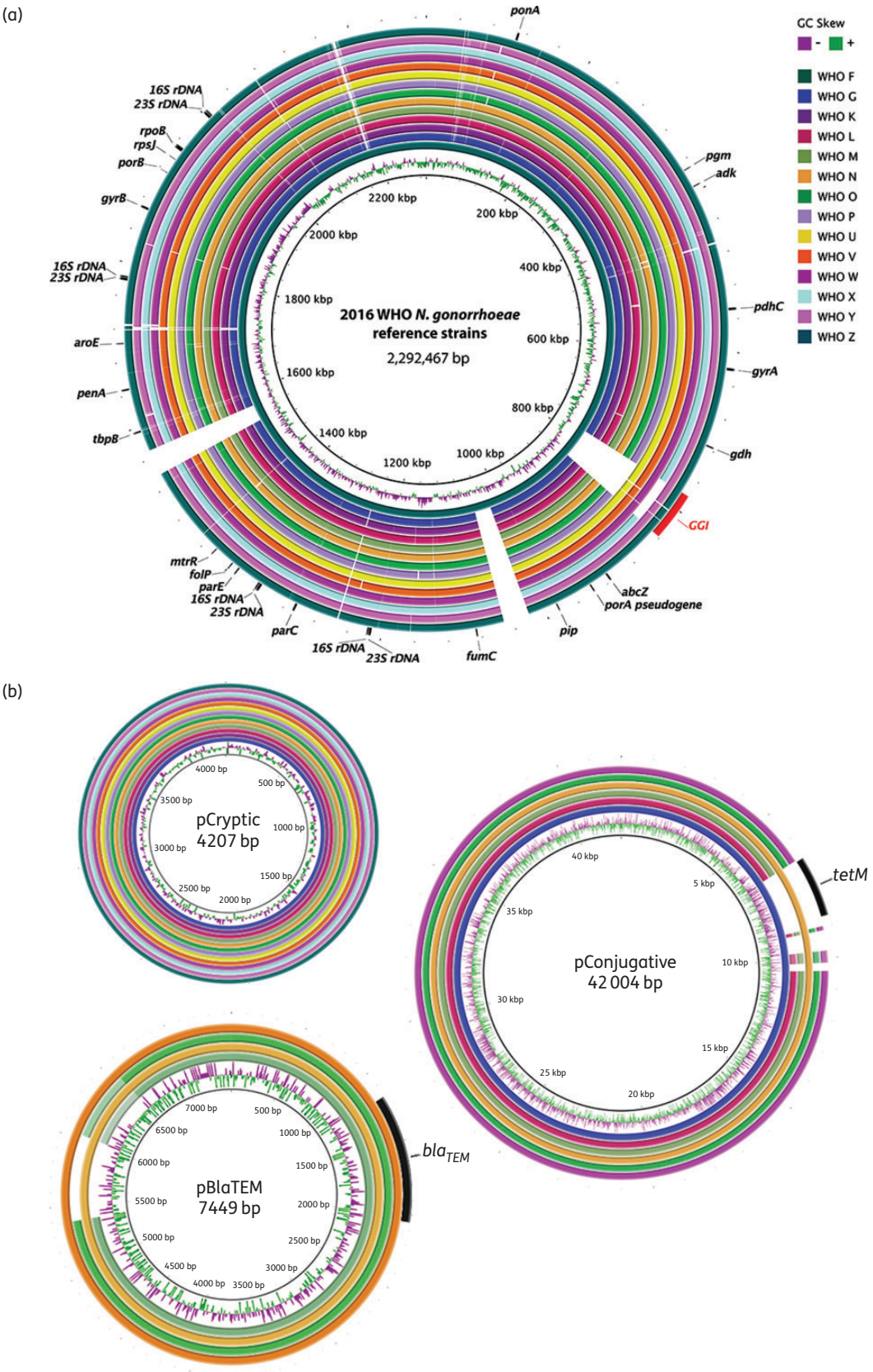
Characteristic	WHO F[a]	WHO G[a]	WHO K[a]	WHO L[a]	WHO M[a]	WHO N[a]	WHO O[a]	WHO P[a]	WHO U	WHO V	WHO W	WHO X	WHO Y	WHO Z
MLST ST	ST10934	ST1903	ST7363	ST1590	ST7367	ST1583	ST1902	ST8127	ST7367	ST10314	ST7363	ST7363	ST1901	ST7363
NG-MAST ST	ST3303	ST621	ST1424	ST1422	ST3304	ST556	ST495	ST3305	ST2382	ST8927	ST835	ST4220	ST1407	ST4015
porA pseudogene mutant[24,25]	—	—	—	—	—	—	—	—	yes	—	—	—	—	—
cppB gene[65–67]	—	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
pip gene mutant[68]	—	yes	—	—	—	yes	—	—	yes	—	—	—	—	—
penA mosaic allele[3,4,69–71]	—	—	yes	—	—	—	—	—			yes	yes	yes	yes
PBP2 A311V[5,70,71]	—	—	—	—	—	—	—	—	—	—	—	yes	—	yes
PBP2 I312M and G545S[69,71]	—	—	yes	—	—	—	—	—	—	—	yes	yes	yes	yes
PBP2 V316T[69,71]	—	—	yes	—	—	—	—	—	—	—	yes	—	yes	yes
PBP2 V316P[5,70,71]	—	—	—	—	—	—	—	—	—	—	—	yes	—	—
PBP2 T483S[4,70,71]	—	—	—	—	—	—	—	—	—	—	—	yes	—	yes
PBP2 A501V[3,4,69,71]	—	—	—	yes	—	—	—	—	—	—	—	—	—	—
PBP2 A501P[3,4,7.71]	—	—	—	—	—	—	—	—	—	—	—	—	yes	—
PBP2 G542S[3,4,71,72]	—	—	—	yes	—	—	—	—	—	yes	—	—	—	—
PBP2 D345 insertion[3,4,71]	—	yes	—	yes	yes	yes	yes	yes	yes	yes	—	—	—	—
PBP2 P551S[3,4,71,72]	—	—	—	—	—	—	yes	—	—	—	—	—	—	—
mtrR promoter; 13 bp inverted repeat[4,71,73–75]	WT	deletion of A	deletion of A	WT	deletion of A	WT	deletion of A	A→C SNP in A-repeat	WT	deletion of A	deletion of A	deletion of A	deletion of A	A→C SNP in A-repeat
MtrR codon G45[4,73–76]	WT	WT	G→D	G→D	G→D	WT	WT	NA[b]	WT	WT	G→D	WT	WT	WT
mtr_{120}[44]	—	—	—	yes	—	—	—	—	—	—	—	—	—	—
mtrR coding region frame shift mutation[4]	—	—	—	—	—	deletion of A at bp 158[b]	—	insertion of T at bp 60[b]	—	—	—	—	—	—
porB1b codon G101[4,71,77,78]	NA[c]	NA[c]	G→K	G→K	G→K	NA[c]	G→K	WT	WT	G→K	G→K	G→K	G→K	G→K
porB1b codon A102[4,71,77,78]	NA[c]	NA[c]	A→D	A→D	A→D	NA[c]	A→D	A→D	WT	A→D	A→D	A→D	A→N	A→D
ponA1; L421→P in PBP1[79]	—	yes	yes	yes	yes	yes	yes	—	yes	yes	yes	yes	yes	yes
gyrA codon S91[4,47,71]	WT	S→F	S→F	S→F	S→F	S→F	WT	WT	WT	S→F	S→F	S→F	S→F	S→F
gyrA codon D95[4,47,71]	WT	WT	D→N	D→N	D→G	D→G	WT	WT	WT	D→G	D→N	D→N	D→G	D→N
parC codon D86[4,47,71]	WT	WT	WT	D→N	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
parC codon S87[4,47,71]	WT	WT	S→R	WT	WT	S→I	WT	WT	S→W	S→R	S→R	S→R	S→R	S→R
parC codon S88[4,47,71]	WT	WT	S→P	S→P	WT	WT	WT	WT	WT	WT	S→P	S→P	WT	S→P
parE codon G410[80]	WT	G→V	WT	WT	WT	G→V	WT	WT	WT	WT	WT	WT	WT	WT
16S rRNA (bp 1192)[d,4,48]	WT	WT	WT	WT	WT	WT	C→T	WT	WT	WT	WT	WT	WT	WT
23S rRNA (bp 2059)[d,4,26]	WT	WT	WT	WT	WT	WT	WT	WT	WT	A→G	WT	WT	WT	WT
23S rRNA (bp 2611)[d,4,49]	WT	WT	WT	WT	WT	WT	WT	WT	C→T	WT	WT	WT	WT	WT
rpoB codon H552[52,71]	WT	WT	WT	WT	H→N	H→N	WT	H→N	WT	WT	WT	WT	WT	WT
rpsJ codon V57[54,71]	WT	V→M	V→M	V→M	V→M	V→M	V→M	V→M	V→M	V→M	V→M	V→M	V→M	V→M
folP codon R228[55,71]	WT	R→S	R→S	WT	R→S	R→S	R→S	R→S	R→S	R→S	R→S	R→S	R→S	R→S
β-Lactamase plasmid type[4,45,46,71]	—	—	—	—	African	Asian	African	—	—	Asian	—	—	—	—
bla_{TEM} allele[46]	—	—	—	—	TEM-1	TEM-1	TEM-1	—	—	TEM-1	—	—	—	—
tet(M) plasmid type[4,53,71]	—	Dutch	—	—	—	Dutch	—	—	—	—	—	—	—	—

[a]Includes some previously published results;[23] however, many additional genes and mutations, and reference genomes, have been characterized in the present paper.
[b]NA, not applicable due to frame-shift mutation that causes a premature stop codon and truncated peptide.
[c]NA, not applicable because these strains were of serogroup WI (PorB1a).
[d]*Escherichia coli* numbering used. Mutations found in all four alleles of the gene.

Table 3. General characteristics of the reference genomes of the 2016 WHO *N. gonorrhoeae* reference strains ($n=14$)

Characteristic	WHO F	WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P	WHO U	WHO V	WHO W	WHO X	WHO Y	WHO Z
Genome size (bp)	2292467	2167361	2169846	2168633	2178344	2172826	2169062	2173861	2234269	2221284	2222386	2171112	2228980	2229351
No. of CDSs	2450	2299	2296	2314	2305	2300	2304	2305	2378	2366	2361	2295	2380	2368
Coding density (%)	84.8	84.5	84.6	84.5	84.5	84.6	84.6	84.6	84.8	84.8	84.7	84.5	84.8	84.6
Average gene size (bp)	793.7	796.5	799.3	791.6	798.7	798.9	796.0	797.6	796.8	795.8	797.5	799.5	794.4	796.7
GC content (%)	52.1	52.6	52.6	52.6	52.6	52.6	52.6	52.6	52.4	52.4	52.4	52.6	52.4	52.4
5S rRNA								4						
16S rRNA								4						
23S rRNA								4						
tRNAs	56	56	57	56	56	56	56	56	56	56	56	57	56	57
ncRNAs	16	15	15	15	15	15	16	16	16	16	16	16	16	16
tmRNA	1	1	1	1	1	1	1	1	1	1	1	1	1	1
No. of genes in pangenome								3478						
No. core genes								1820						
Accessory genes (%)	630 (25.7)	479 (20.8)	476 (20.7)	494 (21.3)	485 (21.0)	480 (20.9)	484 (21.0)	485 (21.0)	558 (23.5)	546 (23.1)	541 (22.9)	475 (20.7)	560 (23.5)	548 (23.1)
DNA uptake sequences (DUSs)[a]	1981 (1533)	1947 (1510)	1950 (1510)	1956 (1518)	1955 (1516)	1951 (1513)	1950 (1519)	1959 (1517)	1963 (1512)	1968 (1518)	1954 (1509)	1949 (1510)	1973 (1522)	1959 (1512)
Number of plasmids	0	2	1	2	3	3	3	1	1	2	2	1	1	1

[a]Total of the 10-mer DUS sequence GCCGTCTGAA (no. of the 12-mer ATGCCGTCTGAA). Note: the 10-mer sequence is included in the 12-mer.


(a)
ponA
16S rDNA
23S rDNA
rpoB
rpsJ
porB
gyrB
16S rDNA
23S rDNA
aroE
penA
tbpB
mtrR
folP
parE
16S rDNA
23S rDNA
parC
16S rDNA
23S rDNA
fumC
pip
abcZ
porA pseudogene
GGI
gdh
gyrA
pdhC
adk
pgm
GC Skew
-
+
WHO F
WHO G
WHO K
WHO L
WHO M
WHO N
WHO O
WHO P
WHO U
WHO V
WHO W
WHO X
WHO Y
WHO Z
2016 WHO N. gonorrhoeae
reference strains
2,292,467 bp
200 kbp
400 kbp
600 kbp
800 kbp
1000 kbp
1200 kbp
1400 kbp
1600 kbp
1800 kbp
2000 kbp
2200 kbp
(b)
pCryptic
4207 bp
500 bp
1000 bp
1500 bp
2000 bp
2500 bp
3000 bp
3500 bp
4000 bp
pConjugative
42 004 bp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
tetM
pBlaTEM
7449 bp
500 bp
1000 bp
1500 bp
2000 bp
2500 bp
3000 bp
3500 bp
4000 bp
4500 bp
5000 bp
5500 bp
6000 bp
6500 bp
7000 bp
blaTEM

characteristic of the *macAB* promoter sequence in gonococci and meningococci and has a dampening effect on the *macAB* transcription compared with a −10 TATAAT sequence.[56] No mutations modulating transcription were found in any of the strains in the putative −35 promoter hexamer sequence (CTGACG) of the promoter sequence for the *norM* gene (encoding the NorM efflux pump) or in its ribosome binding site (TGAA).[57]

Of importance for phenotypic and/or molecular detection of gonococci, *cppB* (WHO F), *pip* (WHO G, N and U) and *porA* (WHO U) mutant strains were represented. Finally, the strains displayed 14 different *porB* alleles, 14 divergent NG-MAST STs and 10 different MLST STs. Notably, the MLST ST7363 and ST1901, and NG-MAST ST1407, were represented, which are internationally spread MDR clones that account for most of the ESC resistance globally (Table 2).[3,4,6,7,10,11,14,19]

Reference genome characterization

The general characteristics of the reference genomes are summarized in Table 3. The genome size ranged from 2 167 361 to 2 292 467 bp. The number of coding sequences (CDSs) after manual curation varied from 2295 to 2450 with an average CDS size of 796.6 bp. The number of core genes was 1820 and accessory genes ranged from 475 to 630. In Figure 1(a and b) BLAST atlases of the 14 reference genomes and all identified plasmid sequences, respectively, are displayed. Briefly, the reference genomes showed relatively high genomic similarity among all strains with the exception of two large insertions in WHO F and the presence or absence of the gonococcal genomic island (GGI).[58] The insertions in WHO F each include 34 almost identical predicted CDSs (31 984 bp) mainly containing an apparently complete type IV secretion system, a *vapD* virulence gene and *xerC* recombinase inserted into a tRNA-Asn element, with only weak matches to other sequences in the NCBI non-redundant nt database. Other regions with low genetic conservation corresponded to mobile genetic elements and prophages (Figure 1a).

Figure 2 shows the phylogenetic relationship among all the reference core genomes ($n=14$, 1820 loci). The number of SNPs between the core genomes is shown on the branches. The average pairwise SNP distance was estimated as 2962 SNPs, with WHO X and WHO Z being the most similar strains (272 SNPs) and WHO F and WHO U the most distant (4969 SNPs). Figure S1 and Table S2, show the pairwise SNP distances among all 14 reference core genomes.

Between none and three plasmids were detected in each strain (Table 3), either from the PacBio or Illumina assemblies. The cryptic plasmid was found in all strains except WHO F, and the β-lactamase plasmid containing a bla_{TEM-1} gene was found in four of the isolates (WHO M, N, O and V; Figure 1b), producing plasmid-mediated high-level penicillin resistance (Tables 1 and 2). The *tet(M)*-carrying conjugative plasmid causing high-level tetracycline resistance through the *tet(M)* gene was found in WHO G and WHO N (Tables 1 and 2). However, WGS also identified the conjugative plasmid in four additional strains (WHO L, M, O and W), although these were lacking the *tet(M)* resistance gene (Figure 1b).

Discussion

In this study, the 2016 WHO *N. gonorrhoeae* reference strains and their detailed phenotypic, genetic and reference genome characteristics are reported. The utility of these strains includes quality control and quality assurance practices in the WHO global and other GASPs. Comprehensive description regarding applications and use of WHO reference strains in GASPs has previously been published.[23,59] The strains include all important susceptible, intermediate susceptible and resistant phenotypes and the ranges of resistances seen for most antimicrobials previously or currently recommended in different guidelines and/or used in the gonorrhoea treatment globally. However, the consensus MIC values (Table 1) were determined using one AMR method only. Accordingly, these MIC values may differ slightly using other AMR methods, though the resistance phenotypes should be consistent. It is strongly recommended that laboratories using the superseded WHO A–E reference strains or the 2008 WHO gonococcal reference strains[23] update to the current 2016 panel. The 2016 WHO gonococcal reference strains will be available through WHO sources and the NCTC (www.phe-culturecollections.org.uk).

In many countries, NAATs are replacing culture for gonococcal detection and, accordingly, genetic detection of AMR determinants to predict resistance or susceptibility to antimicrobials has become of increased interest, both for future AMR surveillance and, ideally, also to guide individually tailored treatment.[4,60,61] Thus, the genetic AMR determinants, acting singly or collaboratively, that mediate the different AMR phenotypes in the 2016 WHO gonococcal reference strains were characterized in detail and included most known gonococcal AMR determinants. These reference strains are designed for internal and external quality assurance and quality control components of both gonococcal phenotypic AMR surveillance and future surveillance using molecular AMR prediction. Molecular AMR methods can never entirely replace phenotypic AMR testing because they only detect identified AMR determinants and new ones will continue to evolve. However, the molecular prediction of AMR or susceptibility can supplement the conventional culture-based phenotypic AMR surveillance. For example, ciprofloxacin susceptibility is relatively straightforward to predict, azithromycin resistance can be indicated and detection of a mosaic *penA* gene can predict decreased susceptibility or resistance to ESCs.[4,60–62] However, due to the many different genes, mutations and accumulation of mutations causing, for example, ESC resistance the molecular methods will not be able to predict an exact MIC of the antimicrobials. However,

Figure 1. BLAST atlas of the 2016 WHO *N. gonorrhoeae* reference genomes ($n=14$). (a) A genome comparison of the 2016 WHO reference strains presented in this study using WHO F[23] as reference and (b) a comparison of the up to three plasmids (named pCryptic, pBlaTEM and pConjugative[65,81,82]) identified in the same strains. WHO G pCryptic (cryptic plasmid), WHO M pBlaTEM (β-lactamase-producing plasmid) and WHO G pConjugative [conjugative plasmid including *tet(M)* in WHO G and N] were used as references, respectively. For each, GC skew is shown in the inner rings. The position in the genomes is shown for genetic resistance determinants and loci used for molecular diagnostics and epidemiological characterization, i.e. NG-MAST STs and MLST STs. The presence of the GGI[58] is indicated in red. An approximately 500 bp region with lower nucleotide conservation (~75% identity) is shown with lighter colours in WHO M and WHO O pBlaTEM plasmids corresponding to a hypothetical protein.

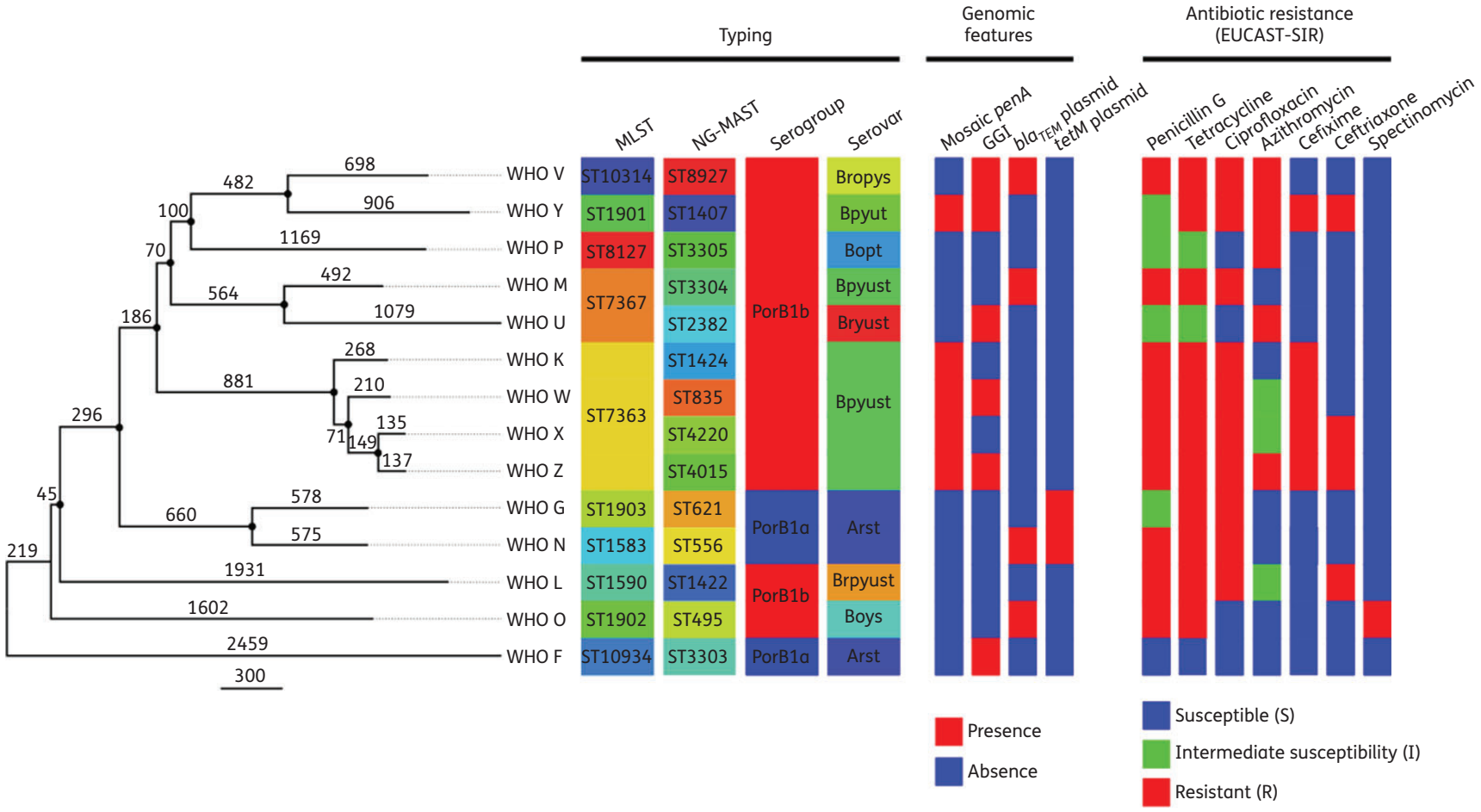


Figure 2. Phylogenetic tree of the 2016 WHO *N. gonorrhoeae* reference core genomes ($n = 14$). The tree is rooted using an *N. meningitidis* genome as outgroup (not shown). The number of SNPs is shown on each branch. Highlighted nodes show bootstrap supports higher than 80%. Typing, genomic features and antibiotic resistance patterns of the 2016 WHO reference strains are shown alongside the tree. Only antimicrobials with a SIR categorization assigned are displayed.

this is not essential if the susceptibility/resistance phenotypes can be predicted by targeting the main AMR determinants. The sensitivity and/or specificity of the AMR prediction will also vary in different settings due to the myriad of gonococcal strains circulating and some cross-reactivity with non-gonococcal *Neisseria* species, particularly in pharyngeal specimens, might be unavoidable.[3,4,60–62] Despite these limitations, molecular prediction of AMR or susceptibility enables testing of substantially more gonococcal samples (including NAAT samples), assessing the spread of genetic potential for AMR development and identifying settings where targeted culture-based phenotypic AMR testing should be initiated. WGS and other novel molecular technologies will likely revolutionize the molecular AMR prediction in gonococci. Ultimately, point-of-care (POC) genetic AMR methods, combined with gonococcal detection, might be used to guide individually tailored treatment of gonorrhoea, which can ensure rational use of antimicrobials (including sparing last-line antimicrobials) and affect the control of both gonorrhoea and AMR.

In recent years, WGS, providing a dramatic increase in resolution, has become more cost-effective and user-friendly. WGS has the potential to revolutionize investigations into gonococcal evolution and population genetics, to identify and track specific strains spreading globally in particular populations and/or in core groups, to identify temporal and geographical changes in strain types as well as the emergence and transmission of individual strains (e.g. MDR ones), to investigate strain identity in contact tracing, test-of-cure and suspected treatment failures, to confirm presumed epidemiological connections or discount isolates from suspected clusters and outbreaks, and to predict AMR or susceptibility in future AMR surveillance.[63] However, when WGS becomes widely used internationally it is crucial that appropriate, validated and finished gonococcal reference genomes are available. Consequently, we present the fully characterized, annotated and finished reference genomes of the 2016 WHO gonococcal reference strains, to enable quality assurance of *N. gonorrhoeae* WGS analysis.

The 2016 WHO *N. gonorrhoeae* reference strain panel includes the previously published 2008 WHO gonococcal reference strains ($n=8$).[23] However, in the present study these strains were subjected to further exceedingly detailed analyses. For example, the susceptibility/resistance to additional antimicrobials (sulfamethoxazole, chloramphenicol, gemifloxacin, moxifloxacin, solithromycin, zoliflodacin, fosfomycin, temocillin and thiamphenicol), additional molecular AMR or diagnostic determinants (mutations in the *rpsJ*,[54] *folP*,[55] 23S rRNA,[4,26,49] *rplD*, *rplV* and bla_{TEM} genes[4,46] and additional *penA* mutations of interest for ESC resistance,[3,4] as well as the presence of *macAB*[56] and *norM*[57] promoter mutations, the mtr_{120} mutation,[44] *ermA*, *ermB*, *ermC* and *ermF* genes,[51,64] *ereA* and *ereB* genes, *mefA/E* genes[50] that can cause macrolide resistance, the *cppB* gene,[65–67] the mutated *porA* pseudogene,[24,25] the mutated *pip* gene[68] and the GGI[58]) and further molecular epidemiological characteristics (MLST STs) were investigated. Furthermore, finished reference genomes were produced, fully annotated and characterized. The six novel WHO reference strains (WHO U, V, W, X, Y and Z) represent phenotypes and genotypes not available when the earlier reference strain panel[23] was developed. Now included are gonococcal strains with low-level to high-level ESC resistance due to different ESC resistance *penA* mutations and associated with both cefixime and ceftriaxone treatment failures. These will be of particular value for enhanced validation of the phenotypic AMR testing, especially monitoring ESC MIC drifts over time. The 2016 WHO reference strains also include a gonococcal strain with high-level azithromycin resistance, due to the A2059G mutation in all four alleles of the 23S rRNA gene,[26] and one with a mutated *porA* pseudogene (*N. meningitidis porA* gene sequences resulting in false-negative results in *porA*-based gonococcal NAATs).[24,25]

In conclusion, the 2016 WHO *N. gonorrhoeae* reference strains were extensively characterized both phenotypically and genetically, including characterizing the reference genomes, and are intended for internal and external quality assurance and quality control purposes in laboratory investigations. These strains should prove particularly useful in WHO global and other GASPs (to allow valid intra- and inter-laboratory comparisons of AMR data derived by different methods in various countries), but also in phenotypic (e.g. culture, species determination) and molecular diagnostics, genetic AMR detection, molecular epidemiology and as fully characterized, annotated and finished reference genomes in WGS analysis, transcriptomics, proteomics and other molecular technologies and data analysis. When additional resistant phenotypes and/or genotypes emerge, novel WHO gonococcal reference strains will be selected, characterized and added to the panel of existing strains.

Acknowledgements

We are very grateful to Janice Lo and Patrice Sednaoui for providing the WHO W strain and the WHO Y strain, respectively.

Funding

This study was supported by grants from: the Department of Reproductive Health and Research, WHO Headquarters, Geneva, Switzerland; the Australian Government Department of Health and the Department of Microbiology, South Eastern Area Laboratory Services, New South Wales, Australia; and Örebro County Council Research Committee and Foundation for Medical Research at Örebro University Hospital, Sweden. WGS and analysis at the Wellcome Trust Sanger Institute was supported through Wellcome Trust grant number 098051. Y. G. was supported by K08-AI104767 from the National Institutes of Health/National Institute of Allergy and Infectious Diseases.

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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