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Recent dynamics in *Neisseria gonorrhoeae* genomic epidemiology in Brazil: antimicrobial resistance and genomic lineages in 2017–20 compared to 2015–16

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Objectives: Regular quality-assured WGS with antimicrobial resistance (AMR) and epidemiological data of patients is imperative to elucidate the shifting gonorrhoea epidemiology, nationally and internationally. We describe the dynamics of the gonococcal population in 11 cities in Brazil between 2017 and 2020 and elucidate emerging and disappearing gonococcal lineages associated with AMR, compare to Brazilian WGS and AMR data from 2015 to 2016, and explain recent changes in gonococcal AMR and gonorrhoea epidemiology.

Methods: WGS was performed using Illumina NextSeq 550 and genomes of 623 gonococcal isolates were used for downstream analysis. Molecular typing and AMR determinants were obtained and links between genomic lineages and AMR (determined by agar dilution/Etest) examined.

Results: Azithromycin resistance (15.6%, 97/623) had substantially increased and was mainly explained by clonal expansions of strains with 23S rRNA C2611T (mostly NG-STAR CC124) and *mtr* mosaics (mostly NG-STAR CC63, MLST ST9363). Resistance to ceftriaxone and cefixime remained at the same levels as in 2015–16, i.e. at 0% and 0.2% (1/623), respectively. Regarding novel gonorrhoea treatments, no known zoliflodacin-resistance *gyrB* mutations or gepotidacin-resistance *gyrA* mutations were found. Genomic lineages and sublineages showed a phylogenomic shift from sublineage A5 to sublineages A1–A4, while isolates within lineage B remained diverse in Brazil.

Conclusions: Azithromycin resistance, mainly caused by 23S rRNA C2611T and *mtrD* mosaics/semi-mosaics, had substantially increased in Brazil. This mostly low-level azithromycin resistance may threaten the recommended ceftriaxone-azithromycin therapy, but the lack of ceftriaxone resistance is encouraging. Enhanced gonococcal AMR surveillance, including WGS, is imperative in Brazil and other Latin American and Caribbean countries.

Introduction

Gonorrhoea and antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* remain significant global public health concerns.[1–3] *N. gonorrhoeae* has a long history of adaptation and development of AMR to all first-line empiric therapies introduced over the years.[3,4] Currently, the first-line treatment for uncomplicated gonorrhoea is ceftriaxone, an extended-spectrum cephalosporin (ESC), either in a high-dose monotherapy or combined with azithromycin.[5–8] However, ceftriaxone, the last remaining treatment option, is also threatened due to resistance emergence internationally.[3–5,9] For example, the ceftriaxone-resistant strain FC428 (carrying mosaic *penA*-60)[10–17] has spread internationally since 2015 and some other sporadic mosaic *penA*-60-carrying strains with ceftriaxone resistance combined with high-level resistance to azithromycin have been confirmed in several countries.[18–20] Furthermore, ceftriaxone-resistant strains carrying mosaic *penA*-60 has also emerged in the more antimicrobial-susceptible genomic lineage B.[21] This worrying spread of ceftriaxone resistance in *N. gonorrhoeae* urges expanded and improved national and international gonococcal antimicrobial surveillance programmes (GASP).[9]

In Brazil, syndromic STI management was introduced in 1993,[22] and this created a lack of etiological diagnostics including culture of gonococci. Consequently, comprehensive AMR data from Brazil were very scarce before the initiation of the national Brazilian GASP in 2015.[23,24] The first Brazilian-GASP data from seven sentinel sites in 2015–16 showed a lack of resistance to ceftriaxone and cefixime, but high prevalence of resistance to ciprofloxacin, tetracycline and benzylpenicillin, and 5.1%–6.9% resistance to azithromycin.[23,24] The high levels of ciprofloxacin resistance across Brazil informed prompt revisions of the national treatment guidelines, i.e. in 2017 ciprofloxacin 500 mg was replaced with ceftriaxone (500 mg) in combination with azithromycin (1 g) as the recommended first-line empirical therapy for uncomplicated gonorrhoea.[22] Furthermore, WGS of the isolates from 2015–16 was used to describe the national genomic baseline for *N. gonorrhoeae* in Brazil;[24] a high proportion belonged to genomic lineage A.[4,24–28] Lineage A includes most strains with resistance to the currently used antimicrobials such as ESCs and azithromycin and the corresponding AMR determinants: mosaic *penA* and *penA* A501 V/T (associated with ESC resistance) and 23S rRNA and mosaic *mtr* (associated with azithromycin resistance).

Understanding the *N. gonorrhoeae* genomic epidemiology and basis of AMR, including the acquisition and dissemination of AMR determinants, is crucial for implementing targeted and evidence-based approaches to combat the escalating threat of AMR in *N. gonorrhoeae*.[4,24–35] The population dynamics and transmission patterns of *N. gonorrhoeae* are complex, driven by various factors such as sexual networks, high-risk populations and social determinants of health. Genomic epidemiology offers not only an unparalleled opportunity to decipher the emergence and spread of AMR, but also the interconnectedness of bacterial strains, providing valuable insights into the routes of transmission, clustering of infections and potential outbreaks.[29,31,33,34]

In the present study, we analysed WGS data in conjunction with AMR data for 623 *N. gonorrhoeae* isolates obtained at 11 sentinel cities across all the five macroregions of Brazil in 2017–20, compared to WGS and AMR data from 2015 to 2016 (seven sentinel sites),[24] to elucidate the circulating gonococcal genomic lineages/sublineages and their AMR determinants, and enhance our understanding of the fluctuations in *N. gonorrhoeae* epidemiology and AMR in Brazil.

Materials and methods

Neisseria gonorrhoeae isolates

N. gonorrhoeae isolates (633; one per patient/gonorrhoea episode) were cultured from consecutive men with urethral discharge attending 11 sentinel cities across Brazil in 2017–20.[36] The men were 18–70 years old, with a median (mean) age of 26 (28) years. The isolates represented all five macroregions in Brazil, i.e. (i) the Northern region: Amazonas (Manaus) $n=97$; (ii) the Northeastern region: Bahia (Salvador) $n=10$ and Pernambuco (Recife) $n=97$; (iii) the Central-Western region: Federal District (Brasília) $n=66$; (iv) the Southeastern region: Minas Gerais (Belo Horizonte) $n=98$ and São Paulo [São Paulo ($n=15$), Ribeirão Preto ($n=95$), São José dos Campos ($n=13$)] and (v) the Southern region: Santa Catarina (Florianópolis) $n=75$, Rio Grande do Sul (Porto Alegre) $n=54$ and Paraná (Curitiba) $n=13$.

In the present study, isolates were after shipment to the WHO Collaborating Centre for Gonorrhoea and Other STIs, Sweden cultured from frozen stocks (−70°C) on GCAGP agar media for 20–24 h in a humid 5% CO_2-enriched atmosphere at 36±1°C, as previously described.[37] If any dubious colony morphology was observed, isolates were verified or falsified as *N. gonorrhoeae* using MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Ten (1.6%) isolates, i.e. from Manaus ($n=3$), Recife ($n=1$), Brasilia ($n=1$), Belo Horizonte ($n=1$) and Porto Alegre ($n=4$), were not viable or verified as *N. gonorrhoeae*. Accordingly, 623 (98.4%) isolates from 2017 ($n=4$), 2018 ($n=85$), 2019 ($n=382$) and 2020 ($n=152$) were available for WGS. One subculturing using identical incubation parameters was performed to ensure the purity of the isolates before DNA extraction.

Antimicrobial susceptibility testing

MICs of eight antimicrobials were determined using agar dilution, in accordance with the CLSI.[38] High MICs of ceftriaxone, cefixime and azithromycin were confirmed using Etest, according to the manufacturer's instructions (bioMérieux, Marcy-l´Etoile, France). Furthermore, isolates with discrepancies between antimicrobial MICs and WGS-derived AMR determinants were also retested using Etest. Where available, EUCAST clinical breakpoints[39] were used to determine susceptibility, susceptibility with increased exposure and resistance. For azithromycin, the epidemiological cut-off (ECOFF) of MIC > 1.0 mg/L[39] was used to indicate isolates with azithromycin-resistance determinants (referred to as azithromycin resistant hereafter). Resistance to ceftriaxone and cefixime was, in accordance with EUCAST clinical breakpoints,[39] defined as MIC > 0.125 mg/L. However, decreased susceptibility (DS) to ceftriaxone and cefixime was also defined, i.e. as MIC = 0.125 mg/L, because isolates with this MIC have previously caused treatment failures.[40–42] The 2016 WHO reference strains[43] were used for quality control of the antimicrobial susceptibility testing.

Whole-genome sequencing and analysis

DNA was extracted using the automated Qiasymphony DSP Virus/Pathogen kit (Qiagen, Hilden, Germany). Illumina DNA Prep (Illumina, Inc. San Diego, CA, USA) libraries were quality controlled using Qubit (Thermo Fischer Scientific, Waltham, MA, USA) and Tapestation (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions before library normalization. Libraries were sequenced on an Illumina NextSeq 550 using Mid Output v.2.5 kits (Illumina) to acquire 149 paired-end reads with an average coverage of >100×.

All reads were quality controlled, trimmed (Phred quality score Q30) and tested for contamination using kmer spectra against the 2016 WHO reference strains[43] with at least 95% mapped reads using customized CLC Genomics Workbench (v.22.0.1) that includes an assembly pipeline as well as *in silico* detection of AMR determinants and typing schemes (MLST (https://pubmlst.org/) and *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR: https://ngstar.canada.ca/)). PyngoST (https://github.com/leosanbu/pyngoST) was used to acquire new alleles for submission to PubMLST and NG-STAR databases and to acquire all NG-STAR clonal complexes (CCs)[44] based on the NG-STAR database (downloaded 5 May 2023). NG-STAR alleles and sequence types (STs) were analysed using goeBURST[45] to explore the correlations between different NG-STAR CCs and AMR determinants using PHYLOViZ v.2.0[46] to generate a minimum spanning tree of isolates from Brazil, 2015–20.

A pipeline to generate a multiple sequence alignment (MSA), phylogenetic tree and additional quality control reports was performed using nullarbor (v.2.0.20191013) (https://github.com/tseemann/nullarbor) with WHO F (accession NZ_LT591897.1)[43] as reference with the following parameters; –ref NZ_LT591897.1.fasta –assembler spades –assembler-opt '–careful' –taxoner kraken2. The MSA was used for phylogenetic inference using IQ-TREE (v.1.6.12)[47] based on 17 907 parsimony-informative sites with subsequent removal of recombinant regions using Gubbins.[48] Simultaneous, phylogenomic analysis as before was performed based on 64 552 parsimony-informative sites using data from Brazil 2015–16 (PRJEB36607),[24] Argentina (PRJEB36608, PRJEB41007)[49,50] as well as the 2016 WHO reference strains (PRJEB14020),[43] WHO R (DRR124693)[10] and WHO Q (ERR2560139)[18] for comparison. The phylogenetic trees were visualized using Microreact.[51]

Isolates from Brazil ($n=623$) were additionally compared to 33 306 publicly available gonococcal genomes (downloaded 31 January 2023) in a global phylogenomic tree, as previously described.[32] This phylogenomic tree was divided into genomic lineages and sublineages using TreeCluster[52] with the average clade mode. The method generated 84 distinct clades in the two main lineages A and B, and clades with >1000 isolates were designated sublineages B1–B7 and A1–A5 (Figure S1, available as Supplementary data at *JAC* Online). Isolates that did not belong to any of these sublineages were designated lineage A or B. Lineage and sublineages for all isolates from Brazil, Argentina and the WHO reference strains ($n=1441$) were redesignated to fit this more stable model of the global gonococcal population for genomic molecular epidemiology. The MSA was used in MEGA11[53] to divide the isolates into the identified lineages and compute the interpopulation diversity and mean distance between the lineages.

An in-house *mtrD* database was used to define mosaic and semi-mosaic *mtrD* variants. The encoded MtrRCDE proteins have regions with high disorder outside the predicted membrane segments suggesting motility. Mosaicism was defined as having recombinations from closely related species in all such regions. We defined semi-mosaicism as mosaic structures in only one of these regions. MtrD semi-mosaics additionally harboured previously described MtrD amino acid alterations associated with increased MICs of azithromycin, i.e. in amino acids R714 and/or K823. All *mtrD* variants and their nucleotide sequences are available through Microreact (https://microreact.org/project/golparian-et-al-mtrd-mosaic).

All raw sequence reads from the present study are available through the European Nucleotide Archive (project accession number PRJEB62806).

Statistical analyses

Statistical analysis of AMR associations with genomic sublineages was performed using logistic regression analysis and Fisher's exact test using statsmodels (v.0.14.0).[54] Statistical significance was defined by $P<0.05$.

Results

Antimicrobial susceptibility in Brazil, 2017–20

The level of resistance in 2017–20 to ciprofloxacin, tetracycline, benzylpenicillin, azithromycin and cefixime was 66.3% (11.6% increase since 2015–16[24]), 40.4% [22.0% decrease; 23.8% had a MIC > 8 mg/L (high-level resistance)], 24.6% (14.5% decrease), 15.6% (10.5% increase) and 0.2% (no changes), respectively (Table S1). The DS to ceftriaxone and cefixime decreased from 0.4% to 0.3% and 6.4% to 4.5%, respectively. No resistance to ceftriaxone or spectinomycin was found, and the highest gentamicin MIC was 16 mg/L.

Molecular epidemiology and antimicrobial resistance determinants

Phylogenomic tree and all metadata are available through Microreact (https://microreact.org/project/6rU79pwTnmd4z9DH3mNtyG-brazil2020). Molecular epidemiology was conducted using genomic epidemiological tools as well as MLST STs and NG-STAR STs and CCs in Brazil.

In total, 93 MLST STs were found among the 623 isolates from Brazil in 2017–20; most common were ST1901 ($n=78$), ST1588 ($n=67$), ST8143 ($n=48$), ST7363 ($n=41$) and ST9363 ($n=32$). Forty-three MLST STs were represented by a single isolate and 15 were new. Furthermore, the isolates were assigned to 232 NG-STAR STs; most prevalent were ST426 ($n=31$), ST1025 ($n=28$), ST442 ($n=25$), ST90 ($n=19$) and ST2148 ($n=17$), and 137 STs were represented by a single isolate and 85 were new. The goeBURST analysis of the NG-STAR STs revealed 65 CCs and six ungroupable isolates. The largest NG-STAR CCs were CC309 ($n=69$), CC426 ($n=57$), CC124 ($n=49$), CC63 ($n=40$) and CC90 ($n=39$). The shifts of MLST and NG-STAR STs and CCs since 2015–16[24] in the Brazil sites are summarized in Table 1. Although MLST ST1901 and ST1588 remained the most common MLST STs, their

Table 1. Number (%) of first and second most common MLST, *Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) STs and NG-STAR CCs in 11 cities across Brazil in 2017–20, compared to 2015–16[24]

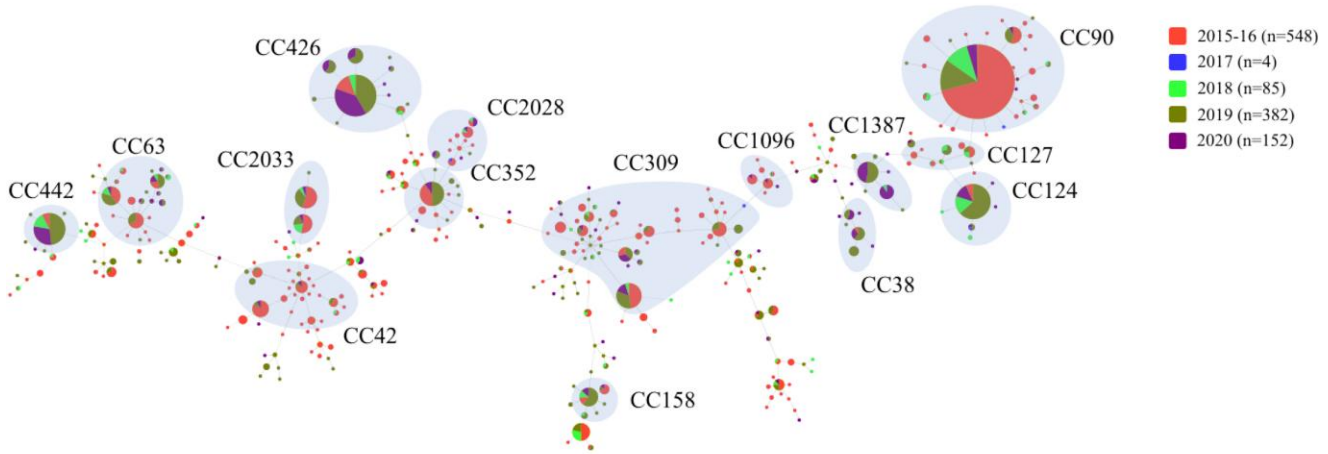
City (2015–16; 2017–20)	MLST ST				NG-STAR ST				NG-STAR CC			
	2015–16		2017–20		2015–16		2017–20		2015–16		2017–20	
	First	Second	First	Second	First	Second	First	Second	First	Second	First	Second
Manaus (*n*=99; 94)	13 429 (21.2)	1901 (17.2)	1588 (25.5)	8143 (19.1)	2217 (11.1)	2092, 2218 (12.1)	426 (17.0)	2072 (8.5)	90 (14.1)	309 (13.1)	426 (20.2)	309 (18.1)
Recife (*n*=0; 96)	NA	NA	1588, 1901 (22.9)	8143, 8156, 13 844 (18.8)	NA	NA	2041, 2079 (12.5)	436, 442 (10.4)	NA	NA	309 (9.4)	426 (8.3)
Salvador (*n*=104; 10)	1588 (27.9)	1901 (15.4)	7827 (20.0)	Singletons (80.0)	90 (12.5)	2079 (8.7)	Singletons (100.0)		309 (21.2)	90 (17.3)	38, 426 (40)	Singletons (60)
Brasilia (*n*=68; 65)	1588 (30.9)	1901 (16.2)	1901, 8156 (24.6)	7827 (7,7)	90 (7.4)	2098 (5.9)	442 (10.8)	2077 (6.2)	309 (19.1)	90 (13.2)	90, 442 (24.6)	1387 (9.2)
Belo Horizonte (*n*=103; 97)	1596 (20.4)	1901 (16.5)	7363 (13.4)	9363 (11.3)	90, 2129 (15.5)	1560 (6.8)	1025 (7.2)	1124, 4379 (12.4)	309 (28.2)	90 (14.6)	309 (15.5)	63, 426 (18.6)
Ribeirão Preto (*n*=0; 95)	NA	NA	1901 (16.8)	7363 (15.8)	NA	NA	1025 (11.6)	158 (10.5)	NA	NA	158 (18.9)	124 (12.6)
São Paulo (*n*=28; 15)	1901 (32.1)	1588 (14.3)	1588, 8143, 12 888 (40)	Singletons (60.0)	1601 (14.3)	90, 439, 1873 (21.4)	2041, 2079 (26.7)	Singletons (73.3)	127 (17.9)	90, 1096 (21.4)	352 (20)	426, 2033 (26.7)
San José dos Campos (*n*=0; 13)	NA	NA	1901 (38.5)	1588 (15.4)	NA	NA	90, 1025 (30.8)	Singletons (69.2)	NA	NA	90, 309 (46.2)	124 (15.4)
Curitiba (*n*=0; 13)	NA	NA	7822 (46.2)	Singletons (53.8)	NA	NA	2148 (38.5)	1387 (30.8)	NA	NA	1387 (69.2)	309 (15.4)
Florianopolis (*n*=74; 75)	1901 (24.3)	1588 (20.3)	1901 (30.7)	8161, 9363 (16)	90 (14.9)	871 (6.8)	90 (9.3)	1025, 4386 (13.3)	90 (23.0)	309 (13.5)	90 (16)	124 (14.7)
Porto Alegre (*n*=72; 50)	8161 (13.9)	13 844 (11.1)	11 602 (14)	8143 (10)	467 (6.9)	2041, 2117 (11.1)	4426 (14)	442, 2086 (12)	352 (16.7)	42 (13.9)	124 (16)	42, 426 (20)
Total (*n*=548; 623)	1901 (17.2)	1588 (16.8)	1901 (12.5)	1588 (10.8)	90 (8.6)	2217 (2)	426 (5.0)	1025 (4.5)	309 (16.8)	90 (15.1)	309 (11.1)	426 (9.1)

NA, not assessed.

Table 2. AMR determinants in 11 cities across Brazil in 2017–20, compared to 2015–16[24]

		City 2017–20 (2015–16)											
Gene	AMR determinant	Manaus 94 (99)	Recife 96 (–)	Salvador 10 (104)	Brasilia 65 (68)	Belo Horizonte 97 (103)	Ribeirão Preto 95 (–)	São Paulo 15 (28)	San José dos Campos 13 (–)	Curitiba 13 (–)	Florianópolis 75 (74)	Porto Alegre 50 (72)	Total 623 (548)
***penA* (%)**	Mosaic	4.3 (22.2)	8.3	0 (15.4)	13.8 (14.7)	8.2 (13.6)	6.3	13.3 (10.7)	15.4	7.7	16.0 (23.0)	10.0 (9.7)	9.0 (16.2)
	A501 V/T	11.7 (2.0)	7.3	30.0 (4.8)	10.8 (13.2)	10.3 (6.8)	18.9	0 (7.1)	23.1	0	4 (0)	10.0 (0)	10.9 (4.6)
23S rRNA gene (%)[a]	C2611T	3.2 (0)	6.3	0 (0)	4.6 (0)	20.6 (4.9)	14.7	6.7 (3.6)	15.4	0	33.3 (4.1)	2.0 (1.4)	12.0 (1.8)
***gyrA* (%)**[b]	S91	74.5 (44.4)	53.1	90.0 (53.8)	61.5 (77.9)	76.3 (67.0)	72.6	40.0 (64.3)	92.3	92.3	56.0 (59.5)	56.0 (22.2)	66.3 (54.7)
***parC* (%)**[d]	D86N	33.0 (0)	5.2	30.0 (2.9)	18.5 (2.9)[c]	16.5 (1.9)	5.3	13.3 (3.6)	0	7.7	6.7 (1.4)	14.0 (2.8)	14.0 (2.0)
	S87	41.5 (37.4)	40.6	60.0 (48.1)	40 (60.3)	45.4 (52.4)	38.9	26.7 (57.1)	84.6	84.6	50.7 (54.1)	42 (13.9)	44.3 (45.3)
	E91	16.0 (15.2)	9.4	30.0 (4.8)	7.7 (26.5)	12.4 (13.6)	35.8	6.7 (17.9)	7.7	0	5.3 (5.4)	6.0 (4.2)	14.0 (11.7)
***ponA* (%)**	L421P	43.6 (32.3)	49.0	70.0 (59.6)	61.5 (73.5)	64.9 (67.0)	70.5	33.3 (71.4)	100.0	84.6	62.7 (58.1)	54.0 (23.6)	59.1 (53.5)
***mtrR* (%)**	–35 A-del. and/or G45D	30.9 (14.1)	29.2	20.0 (28.8)	41.5 (35.7)	30.9 (31.1)	50.5	6.7 (50.0)	61.5	0	42.7 (31.1)	26.0 (13.8)	32.4 (27.2)
	Mosaic	9.6 (3.0)	3.1	10.0 (4.8)	7.7 (0)	9.3 (1.0)	2.1	6.7 (0)	0	0	8.0 (6.8)	0 (0)	5.8 (2.6)
	mtr_{120}	0 (0)	0	0 (1.0)	1.5 (0)	2.0 (3.9)	0	0 (0)	0	0	0 (0)	0 (0)	0.5 (0.9)
***mtrC* (%)**	Mosaic	8.5 (4.0)	3.1	10.0 (2.9)	9.2 (0)	9.3 (0)	2.1	6.7 (3.6)	0	0	8.0 (8.1)	0 (5.6)	5.8 (3.3)
	GC-repeat deletion	2.1 (6.1)	12.5	0 (9.6)	9.2 (22.1)	15.5 (12.6)	8.4	6.7 (7.1)	0	0	9.3 (14.9)	8.0 (13.9)	8.8 (12.4)
***mtrD* (%)**	Semi-mosaic	0 (1.0)	1.0	0 (0)	3.1 (0)	1.0 (0)	0	0 (0)	0	0	1.3 (0)	0 (0)	0.8 (0.2)
	Mosaic	9.6 (3.0)	14.6	10.0 (10.6)	10.8 (7.4)	17.5 (5.8)	3.2	13.3 (7.1)	0	0	12 (12.2)	6 (11.1)	10.4 (8.0)
***mtrE* (%)**	Mosaic	8.5 (4.0)	2.1	0 (3.8)	6.2 (7.4)	9.3 (5.8)	2.1	6.7 (3.6)	0	0	5.3 (17.6)	0 (11.1)	4.8 (1.1)
***porB1b* (%)**	G101/A102	24.5 (33.3)	22.9	30.0 (29.8)	32.3 (38.2)	27.8 (38.8)	26.3	20 (35.7)	53.8	46.2	48.0 (51.4)	28.0 (29.2)	30.0 (36.3)
***rpsJ* (%)**	V57M	98.9 (98.0)	87.5	90.0 (98.1)	83.1 (94.1)	95.9 (98.1)	92.6	93.3 (96.4)	92.3	100.0	88.0 (94.6)	90.0 (95.8)	91.7 (96.7)
***bla*$_{TEM}$ (%)**	β-lactamase	38.3 (20.2)	25.0	20.0 (42.3)	12.3 (41.2)	34.0 (36.9)	21.1	26.7 (25.0)	23.1	7.7	9.3 (17.6)	8.0 (11.1)	22.8 (28.8)
***tetM* (%)**	*tetM*	39.4 (68.7)	32.3	20.0 (59.6)	18.5 (47.1)	30.9 (49.5)	34.7	26.7 (17.9)	23.1	7.7	21.3 (10.8)	20.0 (37.5)	28.7 (46.2)

[a]No isolate with any mutation in 23S rRNA A2058 or A2059 was found.
[b]Two isolates from Recife and one isolate from Manaus had no mutation in S91 but only in D95N.
[c]One isolate had D86G.
[d]No isolate with S88 mutation was found in *parC* in 2017–20.



		CC309 (2015-16)	CC90 (2015-16)	CC63 (2015-16)	CC426 (2015-16)	CC124 (2015-16)	CC352 (2015-16)	CC42 (2015-16)	CC2033 (2015-16)	CC1387 (2015-16)	CC158 (2015-16)
Number of isolates		69 (92)	39 (83)	40 (37)	57 (7)	49 (4)	19 (29)	14 (32)	17 (22)	36 (0)	28 (7)
penA alteration	Mosaic	4 (2)	39 (77)	0 (0)	1 (0)	0 (0)	0 (0)	0 (2)	0 (0)	0	0 (0)
	A501V/T	0 (0)	0 (0)	0 (1)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1	27 (7)
Mtr efflux pump system	A-deletion	0 (0)	37 (74)	0 (0)	1 (0)	41 (4)	0 (0)	0 (0)	0 (0)	0	28 (7)
	G45D	5 (2)	0 (0)	0 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)
	mtrD mosaic	0 (0)	1 (0)	38 (33)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)
	mtrC GC-del[a]	5 (11)	4 (11)	23 (27)	2 (1)	4 (0)	1 (0)	0 (5)	0 (0)	0	0 (0)
penB	Yes	8 (24)	26 (66)	16 (11)	6 (2)	8 (1)	0 (2)	8 (8)	0 (0)	21	18 (7)
	porB1a	22 (29)	5 (5)	4 (6)	36 (5)	37 (2)	0 (2)	1 (5)	1 (2)	12	7 (0)
GyrA S91F		69 (91)	39 (82)	0 (0)	56 (7)	49 (4)	0 (0)	0 (0)	0 (0)	36	28 (7)
ParC alteration(s)		58 (72)	38 (79)	8 (0)	55 (7)	49 (4)	0 (0)	0 (0)	0 (0)	36	27 (7)
ponA1		69 (90)	39 (83)	0 (0)	0 (0)	49 (4)	0 (0)	0 (0)	0 (0)	36	28 (7)
23S rRNA	C2611T	1 (0)	5 (1)	1 (0)	0 (0)	40 (4)	2 (0)	0 (0)	0 (0)	0	0 (0)
	A2059G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)
β-lactamase		47 (73)	0 (4)	0 (0)	33 (4)	0 (0)	1 (1)	1 (5)	2 (2)	0	0 (0)
TetM		51 (76)	0 (1)	1 (1)	0 (0)	1 (0)	0 (6)	14 (31)	17 (22)	0	0 (0)
Median MIC (mg/L)	Ceftriaxone	0.004 (0.008)	0.032 (0.032)	0.008 (0.008)	0.004 (0.004)	0.008 (0.008)	0.004 (0.004)	0.004 (0.004)	0.004 (0.004)	0.016	0.016 (0.016)
	Cefixime	0.008 (0.008)	0.064 (0.064)	0.016 (0.016)	0.008 (0.008)	0.008 (0.008)	0.008 (0.008)	0.008 (0.008)	0.008 (0.008)	0.008	0.032 (0.032)
	Azithromycin	0.064 (0.064)	0.5 (0.25)	0.25 (1)	0.125 (0.125)	4 (16)	0.125 (0.125)	0.064 (0.064)	0.064 (0.064)	0.064	0.125 (0.25)
	Ciprofloxacin	2 (2)	8 (8)	0.004 (0.004)	2 (4)	4 (4)	0.002 (0.004)	0.004 (0.004)	0.004 (0.004)	4	4 (16)
	Tetracycline	16 (16)	1 (2)	0.5 (0.5)	0.5 (0.5)	0.5 (1)	0.5 (0.5)	16 (16)	16 (16)	0.5	2 (2)
	Benzylpenicillin	4 (8)	1 (1)	0.25 (0.25)	16 (32)	1 (1)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	1	0.5 (0.5)
Five-year NG-STAR CC trend		↘	↘	→	↑	↑	↘	↘	↘	↑	↑

Figure 1. The *Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) goeBURST population structure of *N. gonorrhoeae* isolates obtained across Brazil in 2015–20, with relationships illustrated in a minimum spanning tree. There were 289 links with six NG-STAR allele identity and 79 links with five NG-STAR allele identity. The 15 most common NG-STAR clonal complexes (CCs) are illustrated in the minimum spanning tree. The table below the tree shows the AMR determinants for the 10 most common NG-STAR CCs during 2017–20 (2015–16[24]). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

prevalence decreased from 2015–16[24] to 2017–20 by 4.7% and 6.0%, respectively. By contrast, some STs, e.g. ST8143, ST7363 and ST7827 increased by 6.4%, 4.6% and 4.3%, respectively (Figure S2). Shifts in NG-STAR CCs were also observed, for example, CC309 and CC90 that were the most prevalent in 2015–16[24] had decreased in prevalence by 5.7% and 8.9%, respectively. While CC426, CC124 and CC442 instead had increased by 7.9%, 7.1% and 4.6%, respectively (Figure S3). Furthermore, NG-STAR CC38 and CC1387 had emerged in 2017–20 (Figure 1).

AMR determinants decreasing ESC susceptibility such as mosaic *penA*, *penA* encoding A501 substitutions, *mtrR* and *porB1b* were found in 9.0%, 10.9%, 35.6% and 30.0%, respectively, of isolates (Table 2). Forty-one *penA* alleles (seven new) were found, of which six were mosaic. The most common *penA* alleles were *penA*-2.001/2.002/2.006/2.008/2.059 ($n=224$), *penA*-5.002/5.016 ($n=88$) and *penA*-19.001 ($n=83$). Mosaic *penA* or *penA* encoding A501 alteration [*penA*-34.001 ($n=42$), 13.001 ($n=30$), 44.001 ($n=30$), 34.007 ($n=10$), 43.002 ($n=3$), 44.004 ($n=3$) 103.002 ($n=2$), 34.006 ($n=1$), 34.028 ($n=1$), 219.001 ($n=1$) and 223.001 ($n=1$)] in combination with *mtrR* (–35 A-deletion, G45D and/or mtr_{120} ($n=203$)) and *penB* ($n=187$) were present in 63 (10.1%) of isolates, of which 16 (25.4%) and 2 (3.2%) had resistance or DS to cefixime and ceftriaxone, respectively. Of these 63 isolates, 22 (34.9%) belonged to MLST ST1901 and 25 (39.7%) to NG-STAR CC90, which is in contrast to 2015–16[24] when nearly all isolates with decreased ESC susceptibility belonged to NG-STAR CC90 (94.9%). This is also reflected by the decreased prevalence of mosaic *penA* alleles from 2015–16[24] to 2017–20 (Table 2). The single cefixime-resistant isolate carried mosaic *penA*-34.001 and belonged to NG-STAR CC90.

No isolates with mutations in 23S rRNA A2058 or A2059 were found. However, 75 (12.0%) isolates had 23S rRNA C2611T

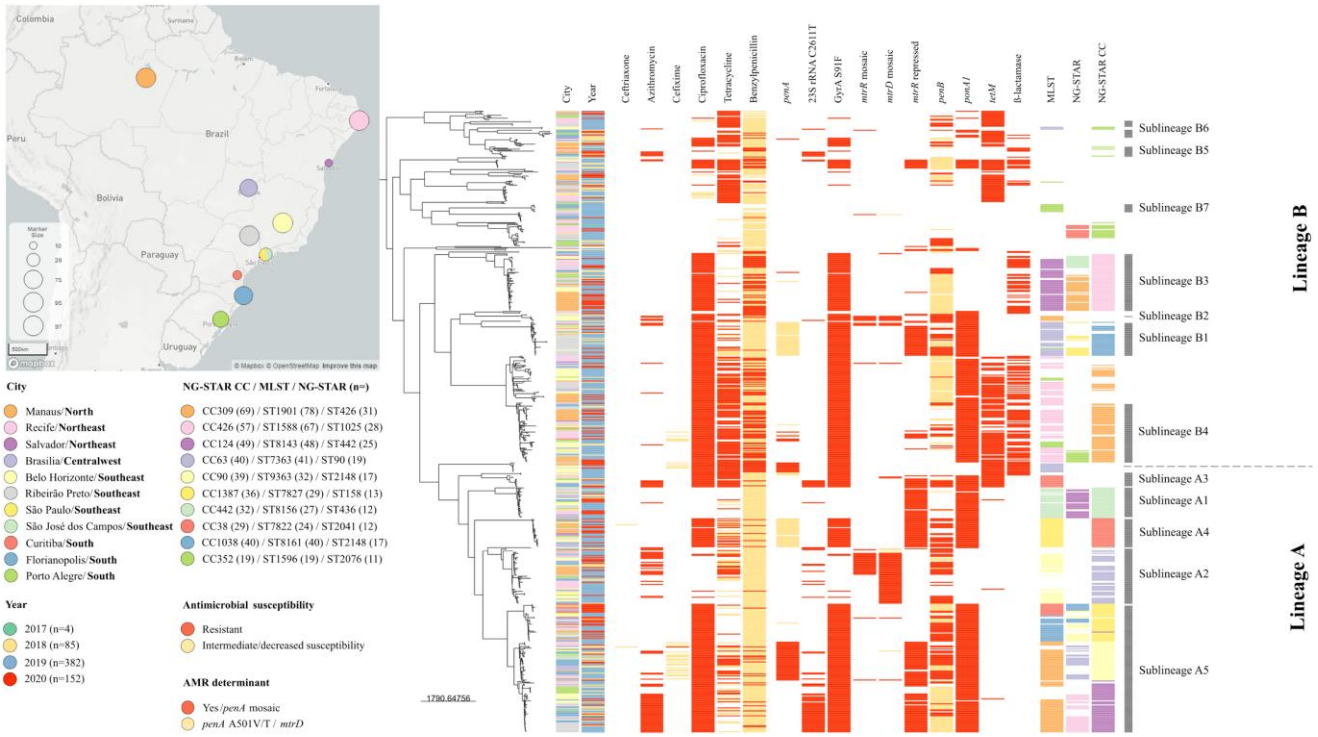


Figure 2. Phylogenomic analysis based on 17 907 parsimony-informative sites. *Neisseria gonorrhoeae* isolates obtained from 11 sentinel cities across Brazil, 2017–20 ($n=623$). The 11 cities are shown on the map. Each node is represented by an isolate, and coloured columns next to the tree are the different metadata and characterized traits. An interactive version of the phylogeography is available through Microreact (https://microreact.org/project/6rU79pwTnmd4z9DH3mNtyG-brazil2020). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

mutations (1–4 mutated alleles) and nine (12.0%) of these 75 isolates also had an *mtrD* mosaic. All these isolates were azithromycin resistant (MIC = 2–64 mg/L), while no azithromycin-susceptible isolates had any 23S rRNA C2611T mutations. The increasing azithromycin resistance due to 23S rRNA C2611T, which was only found in 10 isolates (1.8%) in 2015–16,[24] was primarily caused by the expansion of NG-STAR CC124 (Figure 1, Figure S3) across Brazil. The remaining azithromycin-resistant isolates ($n=22$) had an *mtrD* mosaic ($n=20$) or semi-mosaic ($n=2$). Accordingly, the increase in azithromycin resistance from 5.1% in 2015–16[24] to 15.6% in 2017–20 was caused by the increase in prevalence of 23S rRNA C2611T and *mtrD* mosaics (6.3% and 2.4% increase, respectively) (Table 2). However, 11 and one of the azithromycin-susceptible isolates in 2017–20 also had *mtrD* mosaic and semi-mosaic, respectively. Notably, 6.6% ($n=41$), 11.2% ($n=70$) and 10.0% ($n=62$) of isolates contained mosaic/semi-mosaic *mtrC* (26 alleles), *mtrD* (41 alleles) and/or *mtrE* (16 alleles), respectively. All 34 isolates with mosaic/semi-mosaic *mtrD* alleles (https://microreact.org/project/golparian-et-al-mtrd-mosaic) (13 of 41 *mtrD* alleles) also had mosaic sequence in *mtrR*, *mtrC* and/or *mtrE* with azithromycin MICs ranging from 1–8 mg/L, excluding isolates with 23S rRNA C2611T mutations ($n=9$) or *mtrC* GC hexarepeat deletion ($n=27$), which can increase the susceptibility to azithromycin.[55] Finally, 14 isolates had *rplD* G70D,[56] with no alterations in 23S rRNA or *mtrD* and an azithromycin MIC-range of <0.032–0.5 mg/L.

All ciprofloxacin-resistant isolates carried *gyrA* S91F ($n=413$) and no ciprofloxacin-susceptible isolate had this mutation. The isolates with *gyrA* S91F ($n=413$) also had *gyrA* D95A ($n=248$), D95G ($n=156$) and D95N ($n=9$). Notably, the prevalence of *parC* D86N, which predisposes to emergence of resistance to the new antimicrobial gepotidacin,[57] had dramatically increased from 2.0% in 2015–16[24] to 14.0% in 2017–20 (Table 2).

Plasmid-mediated high-level tetracycline resistance (*tetM*) and chromosomal tetracycline resistance determinant (*rpsJ* V57M) were found in 179 (28.7%) and 571 (91.7%), respectively, of isolates. All tetracycline non-susceptible isolates ($n=326$) had *tetM* ($n=172$) and/or *rpsJ* V57M ($n=320$), beside one isolate with MIC = 1 mg/L. Plasmid-mediated (β-lactamase) high-level penicillin resistance was detected in 142 (22.8%) isolates.

No known mutations causing resistance to zoliflodacin (*gyrB* D429, K450, S467),[32] gepotidacin (*gyrA* A92P),[57] gentamicin (*fusA*)[58] or spectinomycin (16S rRNA, *rpsE*)[3] was found.

Phylogenomic epidemiology in Brazil

The phylogenomic analysis of isolates from Brazil 2017–20 ($n=623$) showed that both genomic lineage A (43.5%) and B (56.5%) of the global gonococcal population (Figure S1) were highly prevalent in Brazil (Figure 2). Most isolates (71.6%) could be designated to one of 12 sublineages (Table S2), i.e. sublineages A1–A5 and B1–B7. Accordingly, 3.7% (10/271) and 44.4% (167/352) of the isolates in lineage A and B, respectively, did not belong to any of the sublineages. The large difference in the proportions of isolates not belonging to any of the sublineages emphasizes

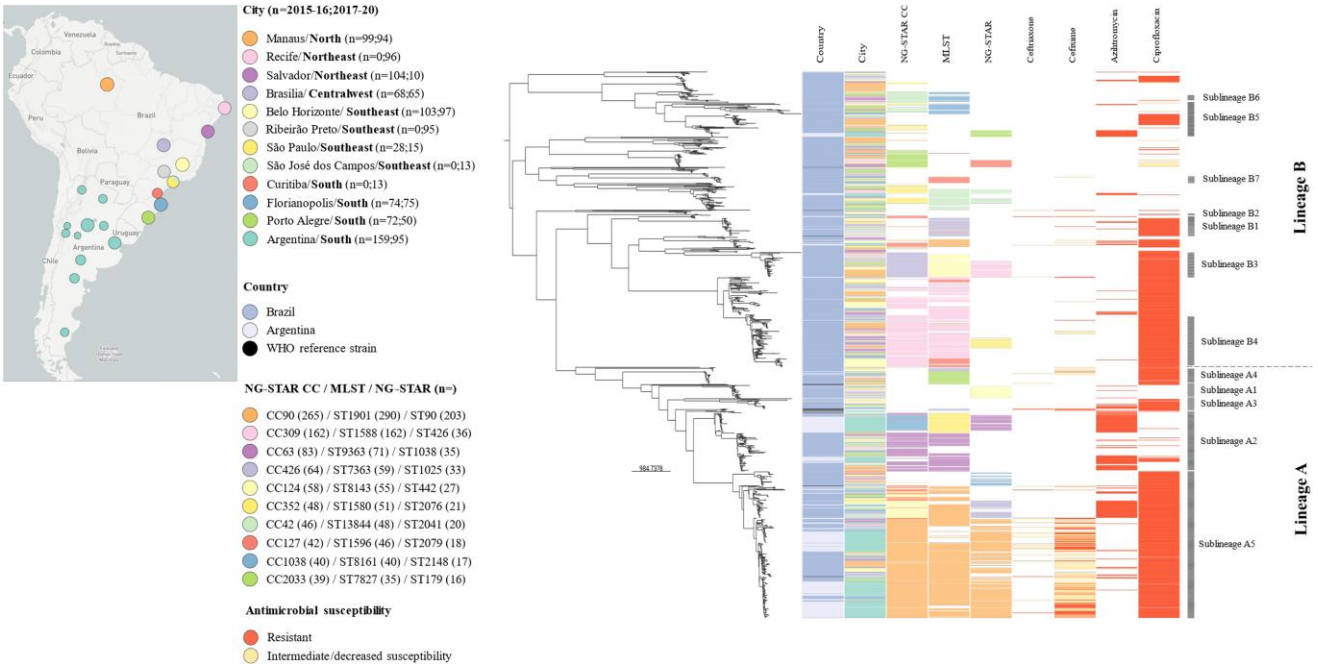


Figure 3. Phylogenomic analysis based on 64 552 parsimony-informative sites. *Neisseria gonorrhoeae* isolates obtained from 11 sentinel cities in Brazil during 2017–20 ($n=623$) are shown in comparison to isolates from Brazil in 2015–16 ($n=548$),[24] isolates with resistance or decreased susceptibility to ESCs ($n=158$)[49] and azithromycin ($n=96$)[50] cultured across Argentina in 2005–19. The 2016 WHO reference strains ($n=14$)[43] are also included. Each node is represented by an isolate, and coloured columns next to the tree are the different metadata and characterized traits. An interactive version of the phylogeography is available through Microreact (https://microreact.org/project/wi2bK9qZ5EzG2ti7ieoefq-brazil16-20arg). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

the substantially higher diversity within lineage B. This was further confirmed by the mean diversity within the lineages; lineage B [5595 single nucleotide polymorphisms (SNPs)] and lineage A (4144 SNPs).

The most common sublineages within lineage A were A5 ($n=130$), A2 ($n=56$) and A1 ($n=31$), and within linage B the most prevalent ones were B4 ($n=60$), B3 ($n=58$) and B1 ($n=34$). Using logistic regression analysis, azithromycin resistance was associated with sublineages A2, A3 and A5, and Fisher's exact test confirmed these associations ($P=0.004$, $P=0.004$ and $P=0.006$, respectively). Resistance plus DS to cefixime was associated with sublineage A5 ($P<0.001$). Resistance to several of the outdated antimicrobials were associated with many sublineages, i.e. sublineages A3 ($P=0.015$), A4 ($P=0.002$), A5 ($P<0.001$), B1 ($P<0.001$), B3 ($P<0.001$) and B5 ($P=<0.001$) with ciprofloxacin resistance; sublineages A3 ($P<0.001$), A4 ($P<0.001$), A5 ($P=0.007$), B1 ($P=0.001$), B4 ($P<0.001$) and B5 ($P=0.015$) with tetracycline resistance and sublineages A5 ($P<0.001$), B3 ($P<0.001$) and B4 ($P<0.001$) with benzylpenicillin resistance. Consequently, sublineage A5 was associated with resistance to all these antimicrobials.

Regarding AMR determinants, 23S rRNA C2611T SNP was associated with sublineages A3 ($P=0.004$), A5 ($P=0.006$) and B5 ($P=0.043$) while *mtrD* mosaics were associated with sublineage A2 ($P<0.001$). Mosaic *penA* was not associated with sublineage A5 ($P=0.083$), despite that the majority (69.0%) of isolates with cefixime DS was within this sublineage. However, *penA* A501V and A501T mutations were associated with sublineages A4 ($P<0.001$) and B1 ($P<0.001$), respectively. Sublineages B4 and B5 were associated ($P<0.001$) with plasmid-mediated resistance to both tetracycline and benzylpenicillin, sublineage A3 with only *tetM* ($P<0.001$), and sublineage B3 with solely β-lactamase ($P<0.001$). Notably, sublineages A4 ($n=29$) and B3 ($n=58$), which were not present in Brazil in 2015–16,[24] with 100% and 77.6% prevalence of *parC* D86N, respectively, were the main reasons for the dramatically increased prevalence of this mutation (Table 2).

Discussion

The present study provides important insights into the levels of AMR and genomic diversity of *N. gonorrhoeae* in Brazil, 2015–20. Increasing resistance to azithromycin and persistently high levels of resistance to ciprofloxacin were observed across Brazil, 2015–20 (Table S1). However, resistance to cefixime remained rare (0.2%), ceftriaxone resistance remained lacking and DS to both ESCs had decreased since 2015–16.[23,24] This decreased ESC DS was due to a reduced prevalence of isolates with mosaic *penA*. However, it is worrying that resistance to azithromycin had increased from 5.1%[24] to 15.6% ($P<0.001$), which correlates especially with the increased prevalence of isolates with 23S rRNA C2611T, i.e. from 1.8%[24] to 12.0% ($P<0.001$) (Table 2). This increase was mainly caused by the rapid expansion of one clade in sublineage A5 belonging to NG-STAR CC124 and MLST1901 (https://microreact.org/project/6rU79pwTnmd4z9DH3mNtyG-brazil2020#1j7l-23srrnac2611t). The spread of this clade was primarily in Southeastern Brazil, and this potentially represents the first successful *N. gonorrhoeae* clonal expansion of a 23S rRNA

C2611T strain. These findings are worrying for the first-line empirical treatment in Brazil (ceftriaxone plus azithromycin)[22] and NG-STAR CC124 should be carefully monitored. Furthermore, mosaic/semi-mosaic *mtrD* alleles causing resistance to azithromycin have also increased in prevalence, from 2.7%[24] to 5.6%, and included two new *mtrD* mosaics (variant 77 and variant 79) and three semi-mosaics (variant 67, 69 and 71) (https://microreact.org/project/ghXEPmnhVr7Svhf8VMPfn1-mtrd-mosaicism). As in Argentina,[50] Europe[33,34] and the USA,[59] the isolates with mosaic *mtrD* primarily belonged to NG-STAR CC63 (MLST ST9363). The increasing azithromycin resistance in gonococci in Brazil (and many other countries) is most probably a consequence of the widespread use of azithromycin therapy to treat *C. trachomatis* and *M. genitalium* infections, non-gonococcal urethritis, respiratory tract infections and general use of macrolides for other infections.[5,60,61] However, it cannot be excluded that the vast azithromycin overuse during the COVID-19 pandemic in Brazil[62] has also contributed to the increasing azithromycin resistance in gonococci.

All ciprofloxacin-resistant isolates had *gyrA* S91F mutations that increased by 11.6% since 2015–16.[24] But the largest increase in ciprofloxacin-resistance determinants was for *parC* D86N, in the two sublineages A4 (NG-STAR CC38) and B3 (NG-STAR CC426) (https://microreact.org/project/6rU79pwTnmd4z9DH3mNtyG-brazil2020#wkos-d86n38426427). Notably, more than half of the isolates were from men who have sex with men in sublineage A4 (51.7%) and 34.9% in sublineage B3. Both NG-STAR CC38 and CC426 had increased ($P<0.001$) from 2015–16 (one and seven isolates, respectively)[24] to 2017–20 (29 and 57 isolates, respectively) (Figure S3). In addition to being involved in ciprofloxacin resistance, the *parC* D86N mutation is predisposing for the emergence of resistance to the novel antimicrobial gepotidacin (GlaxoSmithKline, Brentford, UK); together with zoliflodacin, these are the only two antimicrobials in the later stage of clinical development for gonorrhoea treatment.[57,63–65]

In the present study, gonococcal genomic lineages and sublineages were for the first time defined in a standardized procedure using >33 000 publicly available gonococcal genomes to acquire a global gonococcal phylogenomic structure, which is more stable and accurate. This was used to redesignate lineages and sublineages from Brazil[24] and Argentina.[49,50] Compared to these previous WGS data from Brazil[24] and Argentina[49,50] (Figure 3), the main lineages A (45.9%) and B (54.1%) were found in similar proportions (Figure S4). The gonococcal populations in some sites remained unchanged, while sites in Brasilia and Porto Alegre observed an increase in lineage A isolates. Within Brazil, the phylogenomic shifts that occurred from 2015–16[24] to 2017–20 within lineage A, with more AMR, might be evolutionarily driven by antibiotic use, i.e. from sublineage A5 to sublineages A1–A4 to a higher degree (Table S2). Thus, sublineage A5 was associated with mosaic *penA*-carrying isolates and the strains with ESC DS circulating in Brazil[24] and Argentina during 2013–16,[49] which are now being replaced by azithromycin-resistant strains in sublineages, such as A2, associated ($P<0.001$) with mosaic *mtrD*. Lineage B has remained genomically diverse and less affected by antimicrobial use.[4,25] The fluctuations in the gonococcal population in Brazil are also observed using MLST and NG-STAR CC (Figure S2, Figure S3). MLST ST1901 (NG-STAR CC90) frequently with ESC DS has declined, while ESC-susceptible STs such as ST7827 (NG-STAR CC38), ST8143 (NG-STAR CC426), ST8156 (NG-STAR CC442) have expanded, and ST1588 (NG-STAR CC309) in sublineage B4 has remained stable.

One of the limitations of the present study was that no isolates from women or extra-genital sites were examined. The strengths of the study included the relatively high number of isolates examined by both AMR testing and WGS, and representation of all the five macroregions of Brazil, by surveillance in 11 sentinel cities.

In conclusion, the *N. gonorrhoeae* population in Brazil substantially changed in regard to AMR and genomic structure from 2015–16[24] to 2017–20. Azithromycin resistance, mainly caused by 23S rRNA C2611T and mosaic/semi-mosaic *mtrD*, had substantially increased, however, 56.7% showed low-level azithromycin resistance (MIC = 2–4 mg/L). This mostly low-level azithromycin resistance may threaten the recommended ceftriaxone-azithromycin therapy, but the lack of ceftriaxone resistance is encouraging. Expanded surveillance of gonococcal AMR, including WGS, is imperative in Brazil. Furthermore, enhanced surveillance in other countries in South America, Central America and the Caribbean also remains essential, to gain a more comprehensive understanding of the circulating gonococcal populations, their AMR, factors contributing to this AMR and the dissemination of AMR.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S4 and Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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