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Phylogeny and antimicrobial resistance in *Neisseria gonorrhoeae* isolates from Rio de Janeiro, Brazil

Ana Paula Ramalho da Costa-Lourenço[a], **A. Jeanine Abrams**[b], **Késia Thaís Barros dos Santos**[a], **Isabella Campelo Vilardi Argentino**[a], **Talita Coelho-Souza**[c], **Maria Cristina Albuquerque Caniné**[d], **Adriana Lúcia Pires Ferreira**[d], **Beatriz Meurer Moreira**[a], **Sergio Eduardo Longo Fracalanzza**[a], **David L. Trees**[b], **Raquel Regina Bonelli**[a,*]

[a]Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, CCS, Bloco I, Laboratório I2-59, Cidade Universitária, 21941-902 Rio de Janeiro, RJ, Brazil

[b]National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30329-4027, USA

[c]Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz, Av. Brasil, 4365, Manguinhos, 21040-900 Rio de Janeiro, RJ, Brazil

[d]DASA, Rua Xavier Pinheiro, 439, Parque Duque, 25085-007, Duque de Caxias, RJ, Brazil

Abstract

Resistance in *Neisseria gonorrhoeae* is a global public health challenge. However, little is known about *N. gonorrhoeae* isolates from Brazil. In this study, we characterized 116 *N. gonorrhoeae* isolates obtained in Rio de Janeiro between 2006 and 2015 according to antimicrobial susceptibility profiles, resistance mechanisms, and clonal diversity. We determined antimicrobial minimal inhibitory concentrations by agar dilution, and whole genome sequencing was conducted to investigate alleles related to resistance, determine multilocus sequence typing profiles, and group isolates based on core genome single nucleotide polymorphisms. Resistance to penicillin, tetracycline, ciprofloxacin, and azithromycin was observed since 2006. Resistance to penicillin was mediated by β-lactamase plasmids and chromosomal mutations in *ponA* and *porB* genes, and tetracycline resistance was mediated by TetM plasmids, and *porB* and *rspJ* mutations. Ciprofloxacin resistant isolates presented cumulative point mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC*. Alterations in *rrl* genes encoding 23S *rRNA*, *mtrR*, and the mtrR promoter region were responsible for resistance to azithromycin. Phylogenetic analysis identified seven main clades, which included isolates with similar resistance profiles that mainly belonged to a limited number of sequence types that occurred during different years. Our results demonstrated high penicillin, tetracycline, and ciprofloxacin resistance rates associated

*Corresponding author at: Instituto de Microbiologia Paulo de Goes, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, CCS, Bloco I, Laboratório I2-59, Cidade Universitária, Rio de Janeiro, RJ CEP 21941-902, Brazil. raquel.bonelli@micro.ufrj.br (R.R. Bonelli).

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None declared.

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with the persistence of successful resistant gonococcal lineages, and the polyclonal emergence of azithromycin resistance. Moreover, we reinforce the importance of surveillance to monitor the evolution of this scenario and to allow the early detection of possible changes to azithromycin and ceftriaxone as effective treatment options in the city.

Keywords

Neisseria gonorrhoeae; Antimicrobial resistance; Whole genome sequencing; MLST; Plasmids

1. Introduction

Neisseria gonorrhoeae, the etiological agent of the sexually transmitted disease gonorrhea, is a global concern due to its ability to develop multidrug resistance through many different mechanisms (Unemo and Shafer, 2011). Resistance to low levels of penicillin emerge mainly by cumulative chromosomal mutations in genes encoding penicillin-binding protein 1 (PBP1, *ponA*) and outer membrane protein porin IB (PIB, *porB*) (Olesky et al., 2002; Ropp et al., 2002); whereas the same phenomenon for tetracycline is related to alterations in PIB and in the ribosomal protein S10 (*rpsJ*) (Hu et al., 2005; Olesky et al., 2002). However, high-level resistance to both drugs is achieved through the acquisition of plasmids carrying a β-lactamase (bla) gene type TEM (bla-$_{TEM}$) (Phillips, 1976) or a ribosome protection protein TetM determinant (*tetM*) (Morse et al., 1986). Strains carrying bla$_{TEM}$ plasmids are known as penicillinase-producing *N. gonorrhoeae* (PPNG). These plasmids are genetically related, but have different sizes and insertion sites. Furthermore, the plasmids are named according to their epidemiologic origin as follows: Asia, Africa, Toronto/Rio, Nimes, New Zealand, Johannesburg, and Australian (Trembizki et al., 2014). Two evolutionary unrelated tetM plasmids with similar sizes, named American and Dutch, occur in tetracycline resistant *N. gonorrhoeae* (TRNG) (Turner et al., 1999).

In contrast, resistance to ciprofloxacin, azithromycin, and extended spectrum cephalosporins (ESC), even at high levels, is mediated by chromosomal mutations. Resistance to ciprofloxacin is characterized by cumulative point mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* genes, which encode DNA gyrase and topoisomerase IV, respectively (Giles et al., 2004). Azithromycin resistance in *N. gonorrhoeae* is mainly related to overexpression of the efflux pump MtrCDE and mutations in the drug's target, the V domain of 23S rRNA (Ng et al., 2002). Specific substitutions, A2611G and/or C2599T, in one to four *rrl* alleles encoding 23S rRNA increase azithromycin MIC values in accordance with the number of mutated alleles (Chisholm et al., 2010; Demczuk et al., 2016). Gonococcal ESC resistance is associated with cumulative modifications in PIB and PBP1, overexpression of the efflux pump MtrCDE (*mtrR*), and, especially, mutations in the penicillin-binding protein 2 (*penA*) gene, which encodes PBP2 (Unemo and Nicholas, 2012).

Brazil has recently started a surveillance program for *N. gonorrhoeae*, with no reports published until now. In 2016, the Brazilian Ministry of Health released partial results of this program, indicating high resistance rates of penicillin, tetracycline and ciprofloxacin in the

country (Ministério da Sáude, 2016). Additionally, data associated with this issue are provided by a few sporadic studies performed in specific regions (Belda Junior et al., 2007; Costa et al., 2013; Uehara et al., 2011) or by reports from continental studies, including Brazil; however, little information about sampling details, resistance mechanisms, or the clonal distribution of isolates is included (Dillon et al., 2013; Starnino et al., 2012). These data prompted the Brazilian Health Ministry to publish in September 2017 an update in the recommended therapy for syndromic treatment of gonorrhea, which was previously based in ciprofloxacin combined with azithromycin, to dual therapy with ceftriaxone 500 mg (IM) associated to azithromycin 1 g (PO) in a single dose administration (MS).

Since 2006, the Laboratory for Investigation in Medical Microbiology (LIMM) at the Federal University of Rio de Janeiro has received and analyzed the susceptibility profiles of *N. gonorrhoeae* isolates sent by public and private health care facilities in Rio de Janeiro. In the present study, 116 isolates obtained over almost 10 years (2006–2015) were submitted for whole genome sequencing (WGS), and were subsequently analyzed for antimicrobial susceptibility, resistance mechanisms, and clonal distribution. Here, we provide a detailed description of a Brazilian *N. gonorrhoeae* collection based on clonal and molecular characteristics, which can be used as a reference for comparisons with similar studies performed in other countries.

2. Material and methods

2.1. Sampling

All 116 *N. gonorrhoeae* isolates received by our research laboratory between 2006 and 2015 were studied. These isolates were sent to the LIMM immediately after their isolation and identification by public healthcare facilities and private diagnostic laboratories at their convenience but without any screening. Private laboratories provided 103 *N. gonorrhoeae* isolates. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS (Bruker Biotyper 3.1, Bruker Daltonics) was used to confirm isolate identification. Patient data included specimen type, gender, and age.

The most common specimen type was urethral ($n = 81$), followed by urine ($n = 14$), vaginal ($n = 12$), penile discharge ($n = 3$), cervical ($n = 3$), and rectal specimens ($n = 2$). The specimen type of one isolate was unknown. Ninety-seven patients were male, 15 were female, and four isolates were included in the study without gender identification. Patient ages varied between 13 and 70 years old. Patient sexual orientation is unknown.

2.2. Antimicrobial susceptibility testing

Penicillin, tetracycline, ciprofloxacin, azithromycin, cefixime, and ceftriaxone MIC values were determined by agar dilution (Sigma, US) (CLSI, 2017).

2.3. Whole genome sequencing

Genomic DNA was extracted with the ArchivePure DNA purification kit Protocol 18 (5-PRIME, USA) as described by the manufacturer with the following modifications: 2.2 μL

RNase A solution; 2.5 μL of proteinase K mixed thoroughly by inverting the tube at least 25 times and incubated for 40 min at 37 °C; centrifugation time increased to 6 min for all steps.

WGS was achieved by Illumina HiSeq and MiSeq platforms (2 × 250 bp read lengths). The de novo genome assemblies and the mapping of raw reads to *N. gonorrhoeae* reference strain FA19 (GenBank accession number CP012026) were performed using CLC Genomics Workbench 7 ("CLC Genomics Workbench," n.d.).

2.4. Antimicrobial resistance determinant identification

Mutations conferring resistance to penicillin, tetracycline, quinolones, and azithromycin were identified using CLC Genomics Workbench 7. Specific alterations in PBP1 (L421 K) that lead to increased MICs to penicillin, PIB (G120 K, A121D) mutations related to resistance in penicillin and tetracycline, and S10 Ribosomal Protein (V57 M) associated with resistance to tetracycline (Hu et al., 2005; Olesky et al., 2002; Ropp et al., 2002) were investigated in the translated proteins. To examine alterations in the QRDR, the GyrA and ParC sequences of ciprofloxacin resistant isolates were compared by alignment with protein sequences from the GenBank under accession numbers U08817 (GyrA) and U08907 (ParC). A signal ratio analysis was conducted as previously described (Johnson et al., 2017) to characterize mutations in 23S rRNA. Briefly, SMALT was used to map the sequencing reads of each isolate to an FA19 reference genome that was modified to contain a single copy of 23S rRNA. The ratio of nucleotides at target sites (2059 and 2611 – *Escherichia coli* numbering) was then determined using SAMtools. Mutations in *mtrR* gene and in the *mtrR* promoter region were examined in isolates resistant to at least one antimicrobial.

The presence of plasmids conferring resistance to penicillin and tetracycline was screened by DNA alignment. To detect β-lactamase plasmids (Asia, Africa and Toronto/Rio) (Dillon et al., 1999) and *tetM* (Dutch and American) (Turner et al., 1999) plasmids in WGS data, primers usually applied in PCR reactions targeting such genes were aligned with the genomes to identify fragments that characterize the plasmids. Screening for the bla_{TEM} gene in the β-lactamase plasmids was performed *via* the alignment of the translated protein with the TEM-1 sequence (GenBank accession number NC_019211).

2.5. Multilocus sequence typing

MLST analyses followed the guidelines outlined on the Neisseria Multi Locus Sequence Typing website (PubMLST, n.d.). The sequences of each gene included in MLST analyses were extracted from WGS data using CLC Genomics Workbench 7.

2.6. Phylogenetic analysis

A core genome SNP alignment, based on genomic assemblies, was generated using Parsnp v. 2.5.1. The complete genome of isolate FA19 (Genbank accession number CP012026) was used as a reference. The resulting core genome alignment was then used to reconstruct a maximum likelihood-based phylogeny in RAxML v. 8.0.0 using the general time-reversible (GTR) model with gamma-distributed rate heterogeneity (GTR + Γ) and 1000 bootstrap replicates. Phylogenomic clades were determined based on patristic distances of 0.02

threshold in the phylogenetic tree using RAMI tool (Pommier et al., 2009). Fig. 2 was designed with FigTree v1.4.2 and Phandango ("FigTree," n.d.; Hadfield et al., 2017).

3. Results

3.1. Antimicrobial susceptibility testing

MIC determination by agar dilution revealed yearly resistance rates higher than 40% for penicillin, tetracycline, and ciprofloxacin since 2006 (Fig. 1). Considering both resistance and intermediary resistance, non-susceptibility rates among the 116 isolates reached 99%, 95%, and 67% for penicillin, tetracycline, and ciprofloxacin, respectively (Table 1). Twenty isolates (17%) exhibited azithromycin MICs ≥2 μg/mL, which is the epidemiological cutoff value (ECV) for the occurrence of resistance mechanisms (CLSI, 2017). Furthermore, isolates with azithromycin MIC values ≥2 μg/mL are typically characterized as resistant or moderately resistant (Chisholm et al., 2010; Demczuk et al., 2016; Grad et al., 2014) (Table 1). Azithromycin resistance in *N. gonorrhoeae* in Rio de Janeiro was first observed in 2006, and at least one resistant isolate was detected each subsequent year, with the exception of 2012 (Fig. 1). All isolates were susceptible to ceftriaxone, but seven isolates that exhibited reduced susceptibility to cefixime were identified in different years during the study. A detailed characterization of these strains was previously published (Costa-Lourenço et al., 2017). Table 1 shows the number and percentage of non-susceptible and resistant isolates, MIC_{50}, MIC_{90}, and MIC ranges for each antimicrobial agent.

3.2. Detection of resistance determinants

Whole genome sequences of *N. gonorrhoeae* isolates exhibiting MICs equal to or greater than the resistance cutoff for any antimicrobial were analyzed to investigate associated molecular mechanisms. Typical chromosomal mutations and/or plasmid-mediated resistance mechanisms were identified in most isolates (Table 2). PBP1 L421P, PIB G120 K, and/or PIB A121D substitutions were detected in isolates with penicillin MIC values of 2–16 μg/mL. PPNG isolates harboring Toronto/Rio, Africa, and Asia $bla_{TEM\text{-}1}$ β-lactamase plasmids were associated with MIC values of 4–32 μg/mL. V57 M substitutions in the ribosomal protein S10 that can be associated with PIB G120 K and A121D were observed in isolates with tetracycline MIC values of 2–4 μg/mL. TRNG isolates carrying American and Dutch TetM plasmids presented MIC values of 16–32 μg/mL, with the exception of one strain with an MIC = 2 μg/mL. Two to four cumulative mutations in the QRDR of *gyrA* and *parC* were detected in all ciprofloxacin-resistant isolates. Although no association between specific mutation patterns and ciprofloxacin MICs was observed, the data suggested that mutations in *gyrA* were essential to the development of ciprofloxacin resistance in this dataset, since no isolates presented mutations only in *parC*. Eight azithromycin resistant isolates presented four mutated copies of C2611T in the 23S RNA. Mutations in the mtrR promoter region (−35 A deletion, A → T promoter disruption, and a mosaic-like sequence) were detected in 46 isolates resistant to one or more antimicrobial agents, including those exhibiting reduced susceptibility to cefixime. Four isolates that were resistant to either penicillin, tetracycline, ciprofloxacin, and/or azithromycin revealed mutations in *mtrR* (G45D, A39T, and a premature stop codon). None resistance mechanism was detected in three penicillin resistance isolates (Table 2).

3.3. Typing

A maximum likelihood phylogenetic tree based on whole genome core single nucleotide polymorphisms (SNPs) grouped 78 isolates in seven highly supported clades (Fig. 2), and 38 isolates were distributed in 28 clusters that included up to four isolates. Clades included isolates with similar resistance profiles, which mainly belonged to the same MLST-ST. TRNG and PPNG isolates were also grouped. However, the year of isolation was not a determining factor for clustering (Fig. 2).

Clade A included isolates from ST 1901 ($n = 16$) and ST 11602 ($n = 4$) obtained between 2006 and 2015. Resistance to penicillin, tetracycline, ciprofloxacin, and azithromycin was detected, but these phenotypes were not associated with plasmid carriage. ST 1901 isolates were also grouped in Clades B and F. Clade F ($n = 10$) was composed of isolates resistant to up to four antimicrobials with inconsistent occurrences of β-lactamase and TetM plasmids. Isolates in Clade B ($n = 7$) were first detected in 2010 and included isolates that were resistant to multiple antimicrobials. However, these isolates were largely unassociated with plasmid carriage, with the exception of one PPNG/TRNG isolate. This clade also included the majority of isolates that exhibited reduced susceptibility to cefixime, and this was in contrast to azithromycin resistant isolates that were distributed throughout the tree.

Clades D and E included 21 isolates obtained between 2007 and 2015 with very similar characteristics. All isolates belonged to ST 1588, except for one isolate belonging to ST 1921 in clade G. These isolates were TRNG with an American plasmid and, among them, 17 were PPNG isolates carrying Toronto/Rio ($n = 16$) or Africa ($n = 1$) plasmids. Resistance to ciprofloxacin was detected in 19 isolates. Another clade (G; $n = 9$) was also exclusively composed of TRNG isolates carrying the American plasmid. All isolates belonged to ST 8145 and exhibited resistance to ciprofloxacin.

Clade C contained eleven isolates that were susceptible to all antimicrobials, and these represented three different STs (9363, 8134, and 11,864). The 38 isolates that did not fall into the aforementioned clades exhibited variable antimicrobial susceptibility and plasmid carriage profiles. These isolates belonged to 26 different STs, and 23 ST were unrelated to identified clades. Ten new STs were identified in this study (Fig. 2).

4. Discussion

In this study, we characterized 116 *N. gonorrhoeae* isolates circulating in Rio de Janeiro from 2006 to 2015. Overall, isolates obtained in different years belonged to the same MLST-ST and presented identical resistance determinants, thus suggesting the persistence of a limited number of gonococcal lineages during this period. Additionally, clades revealed by the whole genome core SNP analysis included isolates belonging to one to three MLST types, thus indicating a moderate association between these typing methods.

High resistance rates to penicillin, tetracycline, and ciprofloxacin was observed throughout the study period, and these results support the recommended therapy of ceftriaxone combined with azithromycin to treat gonococcal infections in Rio de Janeiro (CONITEC, 2015). PPNG and TRNG isolates exhibited penicillin and tetracycline MICs greater than

those observed in isolates that exhibited only chromosomal mutations that are associated with the respective drugs. β-lactamase plasmid Toronto/Rio predominated in PPNG isolates, and this contrasts with the results of other studies conducted in South America in which Africa plasmids represented at least 50% of the characterized collections (Dillon et al., 2001; Gianecini et al., 2015a, 2015b). In fact, 16 of 20 isolates carrying Toronto/Rio plasmids in Rio de Janeiro belonged to clades F and G (ST1588), suggesting that the higher incidence of this plasmid type may be a consequence of its association with these successful lineages. The same trend was observed for TetM American plasmids concentrated in clades A, F, and G. Other studies performed in South America only detected Dutch type plasmids among TRNG isolates (Cobo et al., 1999; Dillon et al., 2001).

Cumulative mutations in the QRDR of *gyrA* and *parC* increased the ciprofloxacin MICs as previously reported (Dewi et al., 2004; Su and Lind, 2001). Resistance to ciprofloxacin was associated with all clades, except for clade D that is composed by fully susceptible isolates. Among the 38 isolates that did not belong to any of the clades, 10 were ciprofloxacin resistant and distributed among different STs. Therefore, ciprofloxacin resistance was less prevalent in isolates dispersed throughout the phylogeny (26% resistant) than in isolates associated with the six identified clades (83% resistant). This result suggests the independent evolution of ciprofloxacin resistance in response to the antibiotic therapy guidelines adopted in Brazil over the last 15 years.

High level resistance to azithromycin in *N. gonorrhoeae* (MIC values ranging from 96 to ≥2048 μg/mL) emerged in Argentina in 2001 (Galarza et al., 2009) and drew worldwide attention after reports began emerging from different continents in 2010 (Bercot et al., 2014; Chisholm et al., 2016; Demczuk et al., 2016) In our study, azithromycin resistance MICs ranged from 2 to 16 μg/mL, and were unassociated with specific clades (Fig. 2). Fifteen of the 20 azithromycin resistant isolates identified in this study belonged to three MLST-STs (1901, 1580, and 9363) that were previously identified in the USA, Canada, and Europe (Demczuk et al., 2016; Jacobsson et al., 2016; Papp et al., 2017). Most of the isolates exhibited mutations in the *mtrR* promoter region, including the −35 A promoter deletion or the A → T promoter disruption (Table 2). One isolate had a mosaic-like *mtrR* promoter, which is strongly associated with reduced susceptibility to azithromycin (Grad et al., 2016). Eight isolates exhibited four mutated copies of the 23S rRNA C2611T mutation, including one isolate in Clade A, one isolate in Clade B, two isolates in Clade C, and two isolates that were not associated with any of the target clades. The two remaining isolates were closely related to the fully susceptible isolates in clade D. These results suggest that the C2611T mutations, which are related to higher azithromycin MIC, arose in several lineages, and it is important to note that six of the eight isolates with these mutations were coupled with *mtrR* and *mtrR* promoter mutations.

In contrast to the distribution of azithromycin resistant isolates, all isolates exhibiting reduced susceptibility to cefixime belonged to ST1901 and were mainly concentrated in clade B, with the exception of one isolate located in clade F. However, the mechanism associated with cefixime reduced susceptibility in the isolate found in Clade F differed from the mechanism associated with the six isolates located in clade C (Costa-Lourenço et al., 2017). All seven isolates presented −35 A promoter deletion in the *mtrR* promoter region.

Although composed of all viable isolates received by our laboratory between 2006 and 2015 (without any screening), the dataset examined in the present study is inappropriate for surveillance. Another limitation of the study is the highly variable number of isolates obtained each year, so the data may not reveal subtle resistance trends. Moreover, the sexual orientation of patients, which may impact resistance rates, was unknown. Even so, the study provides a clear picture of molecular resistance mechanisms and circulating lineages. We demonstrated the occurrence of highly resistant lineages that have circulated in Rio de Janeiro since 2006, with the emergence of combined azithromycin resistance and cefixime reduced susceptibility after 2013, which is associated with an internationally relevant ST (1901).

This is the first study to report antimicrobial susceptibility, molecular resistance mechanism, and lineage distribution data with a microand macro-epidemiological perspective with isolates collected in Brazil. Since little is known about gonococcus antimicrobial resistance rates in other regions of the country, our results from Rio de Janeiro reinforce the need to consolidate a national surveillance program for *N. gonorrhoeae* antimicrobial resistance in Brazil.

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The conclusions, findings and opinions expressed by the authors do not necessarily reflect the official position of the US Centers for Disease Control and Prevention (CDC).

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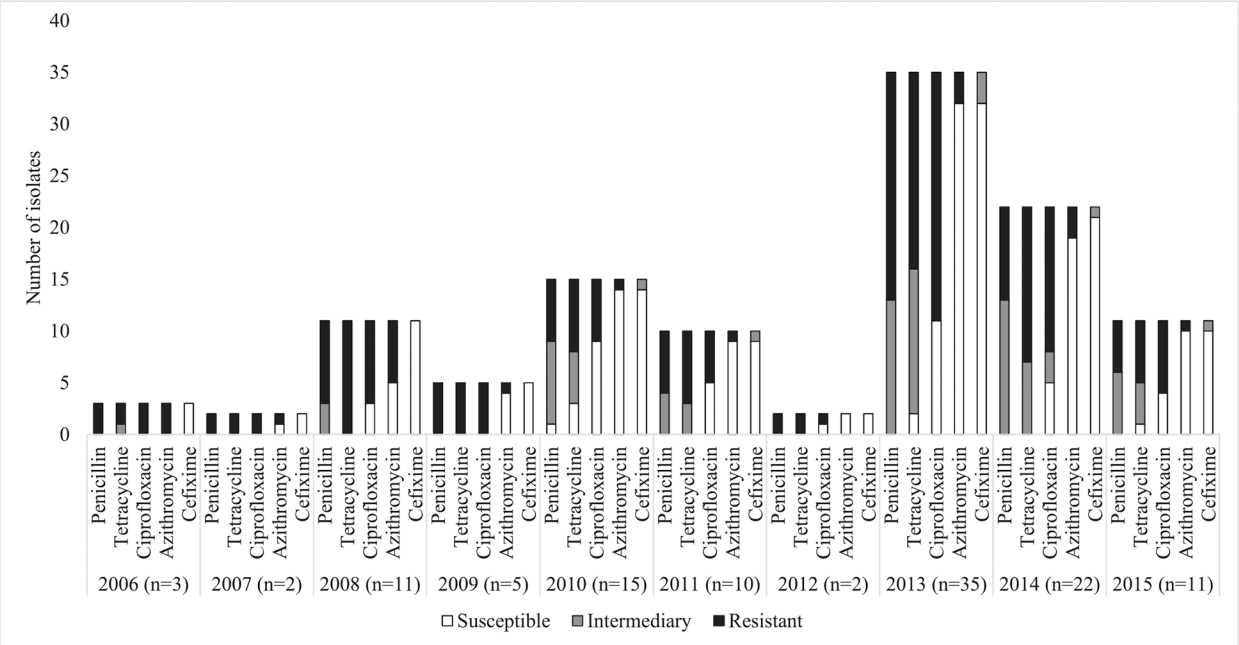

Fig. 1.

Yearly distribution of susceptibility profiles to penicillin, tetracycline, ciprofloxacin, azithromycin, and cefixime of 116 *Neisseria gonorrhoeae* isolates obtained in Rio de Janeiro between 2006 and 2015.

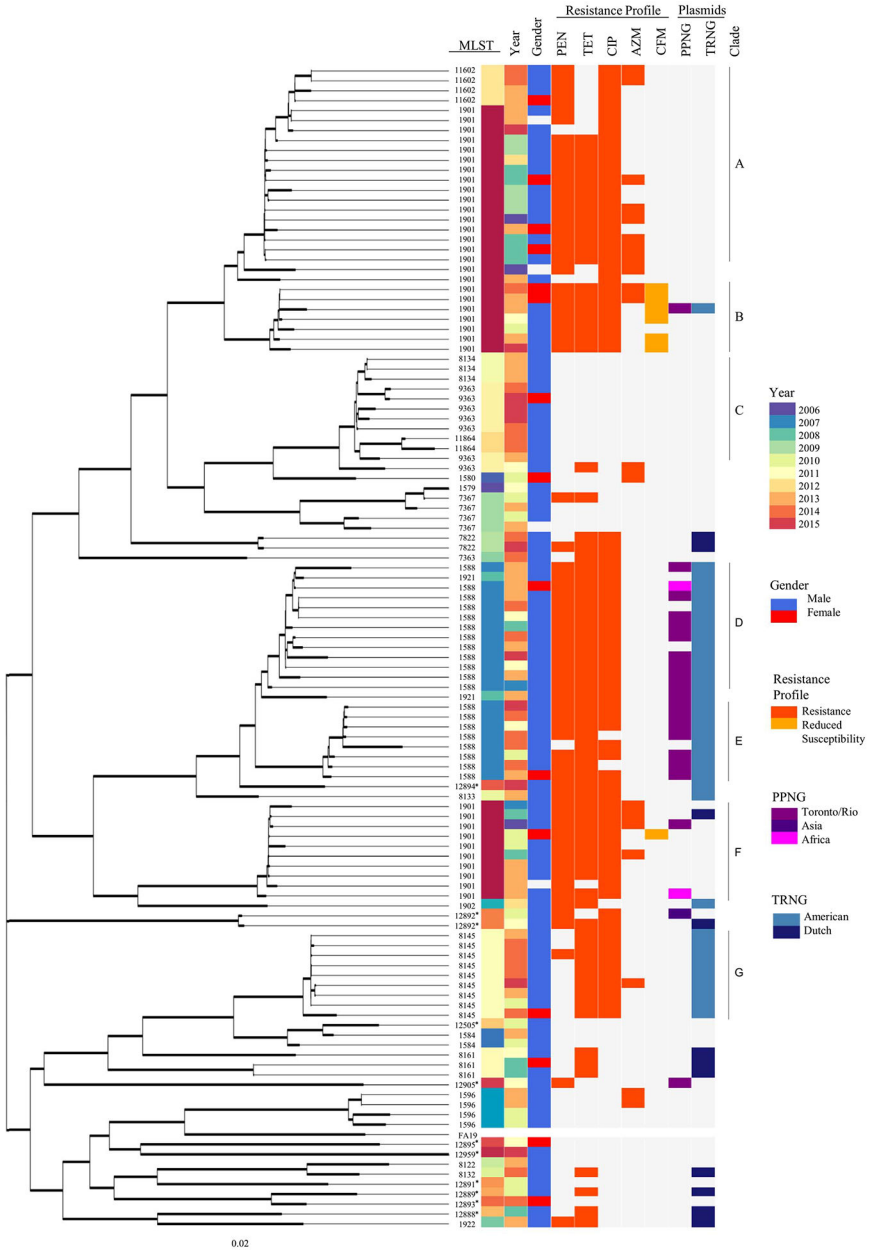

Fig. 2.

Whole genome core SNP maximum likelihood phylogenetic tree of 116 *Neisseria gonorrhoeae* isolates obtained in Rio de Janeiro from 2006 to 2015. Scale bar represents evolutionary divergence of isolates. MLST, year, gender, resistance profile, and plasmid profiles associated with PPNG and TRNG isolates are shown. The 10 new MLST-ST identified in this study are shown with *. Seven distinct clades are highlighted.

Table 1

Antimicrobial resistance and MICs in *Neisseria gonorrhoeae* isolates collected from 2006 to 2015 in Rio de Janeiro.

Antimicrobial	Non-susceptibility (%)[a,b]	Resistance (%)[b]	MIC_{50}	MIC_{90}	MIC range (μg/mL)
PEN	115 (99)	68 (59)	2	16	0.064–32
TET	110 (95)	76 (66)	4	32	0.032–32
CIP	78 (67)	75 (65)	2	16	0.002–32
AZM	54 (47)	20 (17)	0.25	2	0.032–16
CFM[c]	7 (6)	0 (0)	0.016	0.125	0.001–0.25

[a] Includes *N. gonorrhoeae* isolates exhibiting resistance and intermediary resistance to penicillin, tetracycline, and ciprofloxacin; includes *N. gonorrhoeae* isolates with MIC > 0.5 μg/mL to azithromycin.

[b] Percentage was calculated based on total of 116 *N. gonorrhoeae* obtained between 2006 and 2015 in Rio de Janeiro.

[c] Isolates exhibited reduced susceptibility to cefixime as already published (Costa-Lourenço et al., 2017).

Table 2

Resistance determinants identified in *Neisseria gonorrhoeae* isolates resistant to penicillin, tetracycline, ciprofloxacin, and azithromycin.

Antimicrobial agent	Number of resistant isolates	Number of isolates with identified resistance determinants	Type of resistance determinant			
			Chromosomal mutations			Plasmid types
Penicillin	68	65	***porB***	***ponA***	***mtrR***	Toronto/Rio - bla_{TEM-1}
			G120K (27)	L421P (42)	T:A promoter deletion	(20)
			A121D (20)		(38)	Africa - bla_{TEM-1} (2)
					A → T promoter substitution (1)	Asia - bla_{TEM-1} (1)
					G45D (1)	
Tetracycline	76	76	***porB***	***rpsJ***	***mtrR***	American (35)
			G120K (28)	V57M (30)	T:A promoter deletion	Dutch (11)
			A121D (21)		(33)	
					A → C promoter substitution (1)	
					G45D (2)	
Ciprofloxacin	75	75	***gyrA***	***parC***	***mtrR***	
			S91F (74) D95A (30)	D86N (2) S87R (41) E91Q (12)	T:A promoter deletion (41)	
			D95G (44)	S87N (17) E91K (1) S87I (1)	A → C promoter substitution (1)	
					A → T promoter substitution (1)	
					G45D (2)	
Azithromycin	20	20	***rrl* (23S)**		***mtrR***	
			C2611T 4/4 (8)		T:A promoter deletion (14)	
					A → C promoter substitution (1)	
					A → T promoter substitution (1)	
					G45D (1)	
Cefixime[a]	7	7	***penA***		***mtrR***	
			Mosaic *penA* XXXIV	(6) *penA* XII (1)	T:A promoter deletion (7)	

[a]Data previously published (Costa-Lourenço et al., 2017).