International Journal of Infectious Diseases 79 (2019) 116–122

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Antimicrobial resistance and molecular characteristics of *Neisseria gonorrhoeae* isolates from men who have sex with men

Joana Calado[a,1], Rita Castro[a,*], Ângela Lopes[a], Maria José Campos[b], Miguel Rocha[b], Filomena Pereira[c]

[a] *Global Health and Tropical Medicine (GHTM) Medical Microbiology Unit, Institute of Hygiene and Tropical Medicine, New University of Lisbon (UNL), Lisbon, Portugal*
[b] *GAT – Portuguese Non-governmental organization (NGO) of people living with HIV, Lisbon, Portugal*
[c] *Global Health and Tropical Medicine (GHTM) Tropical Clinic Unit, Institute of Hygiene and Tropical Medicine, New University of Lisbon (UNL), Lisbon, Portugal*

ARTICLE INFO

Article history:
Received 9 August 2018
Received in revised form 30 October 2018
Accepted 31 October 2018
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Neisseria gonorrhoeae
Antimicrobial resistance
parC
gyrA
penA
mtrR

ABSTRACT

Objectives: To analyze the susceptibility of *Neisseria gonorrhoeae* isolates to penicillin (Pen), cefixime (Cfm), ceftriaxone (Cro), tetracycline (Tet), ciprofloxacin (Cip), azithromycin (Azm), and spectinomycin (Spt), and to verify the presence of mutations in resistance genes.
Methods: Antibiotic susceptibility testing was performed by Etest method on 30 *N. gonorrhoeae* isolates collected from the MSM (men who have sex with men) population. PCR and DNA sequencing were performed to identify mutations within the *penA*, *mtrR*, *gyrA*, and *parC* genes in intermediately resistant and fully resistant isolates.
Results: N. gonorrhoeae isolates showed intermediate or full resistance to Pen (73%), Cfm (3%), Tet (60%), Cip (37%), and Azm (13%). One isolate with resistance to Cfm presented a penicillin-binding protein 2 (PBP2) mosaic XXXIV. All isolates with intermediate or full resistance to Pen (except at PBP2 mosaic) presented a D345a in PBP2. All Cip-resistant isolates had an S91F in the *gyrA* gene together with mutations in the *parC* gene. All intermediate or fully resistant isolates to substrates of the MtrCDE efflux pump had an A39T or G45D mutation in the *mtrR* gene or an adenine deletion within the *mtrR* promoter. One isolate presented a *Neisseria meningitidis*-like *mtrR* promoter sequence.
Conclusions: The results of this study are consistent with the findings of other studies and reinforce the importance of the expedient development of new therapeutic options.

Introduction

Infections caused by *Neisseria gonorrhoeae* have high epidemic potential and have traditionally shown high levels of antimicrobial resistance, which reduces treatment options and thus increases the chances of the infection becoming incurable. The decreased susceptibility of *N. gonorrhoeae* to extended-spectrum cephalosporins (ESCs) has been reported in Europe. This situation has become an important public health issue, and it is probably only a matter of time before this phenotype becomes widespread in Europe. It is therefore imperative to invest not only in the research of antimicrobial resistance, but also in the development and research of new targets and therapeutic approaches for the treatment of infections caused by this microorganism (ECDC, 2013).

The genetic mechanisms of *N. gonorrhoeae* resistance to antibacterial agents include the acquisition of certain genes and the development of mutations in specific genes and in their regulatory regions (Ilina et al., 2008; Allen et al., 2011). Penicillin resistance mechanisms are related to the acquisition of the *blaTEM-1* gene, the development of mutations in the *penA* and/or *ponA* genes, overexpression of the MtrCDE efflux pump, and *porB* mutations. Tetracycline resistance mechanisms are related to the acquisition of the *tetM* gene, *rpsJ* mutations, overexpression of the MtrCDE efflux pump, and *porB* mutations. Macrolide resistance in *N. gonorrhoeae* may result from several mechanisms, such as specific mutations in the 23S rRNA, the action of rRNA methylases, and overexpression of the MtrCDE efflux pump. The resistance mechanisms developed by *N. gonorrhoeae* to fluoroquinolones are

* Corresponding author at: Instituto de Higiene e Medicina Tropical, Unidade de Microbiologia Médica, Rua da Junqueira No. 100, 1349-008 Lisboa, Portugal.
E-mail address: ritacastro@ihmt.unl.pt (R. Castro).
[1] Present address: Portuguese Oncology Institute (IPO) in Lisbon.

https://doi.org/10.1016/j.ijid.2018.10.030

due to mutations in the *gyrA* and *parC* genes in a well-defined area called the quinolone resistance determining region (QRDR). The resistance of these bacteria to ESCs is mainly due to mutations that modify the action target (penicillin-binding proteins (PBPs)) (*penA* and *ponA* genes) (Kunz et al., 2012; Unemo and Shafer, 2014; Costa-Lourenço et al., 2017).

In men who have sex with men (MSM), gonoorrhoea commonly occurs in extra-genital locations. In addition, most of these infections are asymptomatic. For this reason, this population is one of the main means of transmission of gonorrhoea (ECDC, 2013; Unemo et al., 2013; Costa-Lourenço et al., 2017).

The objectives of this study were to analyze the susceptibility of *N. gonorrhoeae* to penicillin (Pen), cefixime (Cfm), ceftriaxone (Cro), tetracycline (Tet), ciprofloxacin (Cip), azithromycin (Azm), and spectinomycin (Spt) and to verify the presence of mutations in the *penA*, *mtrR*, *gyrA*, and *parC* genes of intermediately resistant and fully resistant isolates.

Materials and methods

N. gonorrhoeae *isolates*

N. gonorrhoeae isolates were obtained by culture of rectal or urethral exudates of MSM attending an HIV and sexually transmitted infections clinic in Lisbon (GAT- CheckPoint) between January 2013 and February 2015. Swabs were inoculated into chocolate agar PolyVitex VCAT3 culture medium (bioMérieux). The identification of *N. gonorrhoeae* was performed according to methods described previously (Unemo et al., 2013). Isolates confirmed to be *N. gonorrhoeae* were stored at −80 °C.

Antimicrobial susceptibility testing

Each isolate was subcultured twice before antimicrobial testing. The Etest method was performed on GC agar (Oxoid) with 1% growth supplement Biovitex (Biolite), according to the manufacturer's instructions. The results obtained were analyzed in accordance with the breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; version 4.0) (EUCAST, 2014). The *N. gonorrhoeae* ATCC 49226 strain was used as quality control. Isolates were classified as penicillinase-producing *N. gonorrhoeae* (PPNG) when β-lactamase activity was detected using a nitrocefin disc (Oxoid).

Molecular studies

DNA was extracted from intermediately resistant and fully resistant isolates using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. When *N. gonorrhoeae* isolates showed intermediate or full resistance to the antibiotics studied, the possible resistance gene or genes were selected for study, as follows: the *penA* gene (PBP2 transpeptidase domain: amino acids 340 to 570) for penicillin-resistant (PenR), penicillin-intermediate (PenI), and cefixime-resistant (CfmR) phenotypes; the *mtrR* gene (MtrR binding domain: amino acids 1 to 60) and 13-bp inverted-region of the *mtrR* promoter sequence for PenR, PenI, tetracycline-intermediate (TetI), tetracycline-resistant (TetR), TRNG (*N. gonorrhoeae* exhibiting high-level tetracycline resistance), and azithromycin-intermediate (AzmI) phenotypes; and the *parC* gene (QRDR region: amino acids 66 to 119) and *gyrA* gene (QRDR region: amino acids 55 to 110) for QRNG (quinolone-resistant *N. gonorrhoeae*) phenotypes. It was chosen to study the *mtrR* gene because it can affect more than one antibiotic and it was not possible to study all of them.

PCR techniques were performed using primers (NZYTech) described previously (Table 1) (Tanaka et al., 2000; Cousin et al., 2003; Perilla et al., 2003; Allen et al., 2011; Liao et al., 2011). The PCR reaction mixture included 10 × Taq buffer (Bioline), 16 μM deoxyribonucleotides (Bioline), 50 mM magnesium chloride (Bioline), 12.5 μM each primer, 5 U/μl of Taq DNA polymerase enzyme, and 5 μl of DNA. All PCR techniques were performed using an Eppendorf Mastercycler Personal machine and the following cycle parameters: 94 °C for 2 min; 40 cycles of 94 °C for 30 s, 48 °C (*penA*), 57 °C (*mtrR/gyrA*), 63 °C (*parC*) for 1 min; 74 °C for 30 s and 74 °C for 5 min. The amplified products were confirmed by electrophoresis on a 1.5% agarose gel and sent to the company STAB VIDA for sequencing, where the same specific primers were used. The BioEdit program and ExPASy, BLAST, and MAFFT online programs were used to analyze the DNA sequences.

Results

The rate of *N. gonorrhoeae* infection in this study was 5.8% (30/518), corresponding to 4.9% (25/508) of the rectal exudate samples and 50.0% (5/10) of the urethral exudate samples during the period January 2013 to February 2015. Symptomatic cases corresponded to 1.9% (10/518) of the population.

The antimicrobial susceptibility testing results are presented in Table 2 and the phenotype classification and associated mutations of the *N. gonorrhoeae* isolates are shown in Table 3.

No multidrug-resistant *N. gonorrhoeae* (MDR-NG) isolates were found in this study (Tapsall et al., 2009). One isolate showed a *Neisseria meningitidis*-like *mtrR* promoter sequence (encoding the *mtrR* promoter and the DNA-binding domain of the MtrR repressor) with 100% nucleotide identity to the *N. meningitidis* strain LNP21362 (GenBank accession number CP006869), confirmed through the BLAST program (Figure 1).

Table 1
Nucleotide sequences of primers and the amplified regions.

Primers	Nucleotide sequence (5′–3′)	GenBank accession number	Amplified region	Reference
penA-F	CGATATGATCGAACCTGG	M32091	nt: 1011 to 1869	Liao et al. (2011)
penA-R	ACAATCTCGTTGATACTCG	(AAA25463)	(aa: 304 to 581[a])	
mtrF1	GCCAATCAACAGGCATTCTTA	Z25796	nt: 853 to 1253	Cousin et al. (2003)
mtr13R1	GTTGGAACAACGCGTCAAAC	(CAA81045)	(aa: 1 to 60[b])	
Ng-gyrA-F	TCCGCCACGACCACAAATTC	U08817	nt: 17 to 433	Allen et al. (2011)
Ng-gyrA-R	CTGCCAGCATTTCATGTGAG	(AAA82128)	(aa: 6 to 144[c])	
Ng-parC-F	GTTTCAGACGGCCAAAAGCCC	U08907	nt: 121 to 420	Allen et al. (2011)
Ng-parC-R	CGGACAACAGCAATTCCGCAAT	(AAA82151)	(aa: 41 to 142[d])	

nt, nucleotides; aa, amino acids; PBP2, penicillin-binding protein 2; QRDR, quinolone resistance determining region.
[a] Transpeptidase domain of PBP2: amino acids 340 to 570 (Liao et al., 2011).
[b] DNA-binding domain of the MtrR subunit: amino acids 1 to 60.
[c] QRDR region of the GyrA subunit: amino acids 55 to 110 (Tanaka et al., 2000).
[d] QRDR region of the ParC subunit: amino acids 66 to 119 (Tanaka et al., 2000).

Table 2
Antimicrobial susceptibility testing results ($n = 30$).

Antibiotic	Concentration range of Etest (mg/l)	Susceptible *n* (%)	Intermediate *n* (%)	Resistant *n* (%)
Penicillin	0.02–32	8 (27)	17 (57)	5 (17)
Cefixime	0.016–256	29 (97)	0 (0)	1 (3)
Ceftriaxone	0.002–32	30 (100)	0 (0)	0 (0)
Tetracycline	0.15–256	16 (53)	9 (30)	5 (17)
Ciprofloxacin	0.002–32	19 (63)	0 (0)	11 (37)
Azithromycin	0.016–256	26 (87)	4 (13)	0 (0)
Spectinomycin	0.064–1024	30 (100)	0 (0)	0 (0)

Table 3
Phenotype classification and associated mutations of *Neisseria gonorrhoeae* isolates ($n = 30$).

Phenotype	PBP2 mutation pattern[a]	MtrR mutations[b]	*mtrR* promoter mutations	GyrA mutations[c]	ParC mutations[d]	Isolate number
PenI	XXXIX	G45D	WT	–	–	NG2
	II	*	–	–	–	NG9
	II	A39T/R44H	WT	–	–	NG28;NG16
	II	A39T	WT	–	–	NG20
	XXII	A39T	T insertion	–	–	NG11
TetI	–	A39T/R44H	WT	–	–	NG14
TetR	–	A39T/Y48D	WT	–	–	NG29
AzmI	–	A39T/R44H	WT	–	–	NG3
PenI; TetI	XII	WT	A deletion	–	–	NG25
	II	A39T/R44H	WT	–	–	NG18; NG19
PenI; TRNG	XIV	A39T/Y48D	WT	–	–	NG13
TetI; AzmI		A39T/R44H	WT	–	–	NG4
PPNG; QRNG	–	–	–	S91F/D95A	S87R	NG21
	–	–	–	S91F/D95A	S87N	NG22; NG23
PenI; TetI; AzmI	IX	WT	A deletion	–	–	NG8
PenI; TetI; QRNG	XXXX	WT	A deletion	S91F/D95G	E91G	NG1; NG6
	II	A39T	WT	S91F/D95A	S87N	NG26
PenI; TetR; QRNG	V	WT	A deletion	S91F/D95G	S87R	NG12
PenI; TRNG; QRNG	XIX	A39T	WT	S91F/D95A	S87N	NG5
PenR; TetI; QRNG	II	A39T	WT	S91F/D95A	D86N	NG27
PenI; TetI; QRNG; CfmR	XXXIV	WT	A deletion	S91F/D95G	S87R	NG17
PenR; TetR; AzmI; QRNG	XXXX	WT	A deletion	S91F/D95G	E91G	NG7

A, adenine; AzmI, intermediate resistance to azithromycin; CfmR, resistant to cefixime; PBP2, penicillin-binding protein 2; PenI, intermediate resistance to penicillin; PenR, resistant to penicillin; PPNG, penicillinase-producing *N. gonorrhoeae*; QRNG, quinolone-resistant *N. gonorrhoeae*; T, thymine; TetI, intermediate resistance to tetracycline; TetR, resistant to tetracycline; TRNG, *N. gonorrhoeae* exhibiting high-level tetracycline resistance; WT, wild-type.

* *Neisseria meningitidis*-like *mtrR* promoter sequence.

[a] Amino acids 340 to 575.

[b] Amino acids 1 to 60.

[c] Amino acids 55 to 110.

[d] Amino acids 66 to 119.

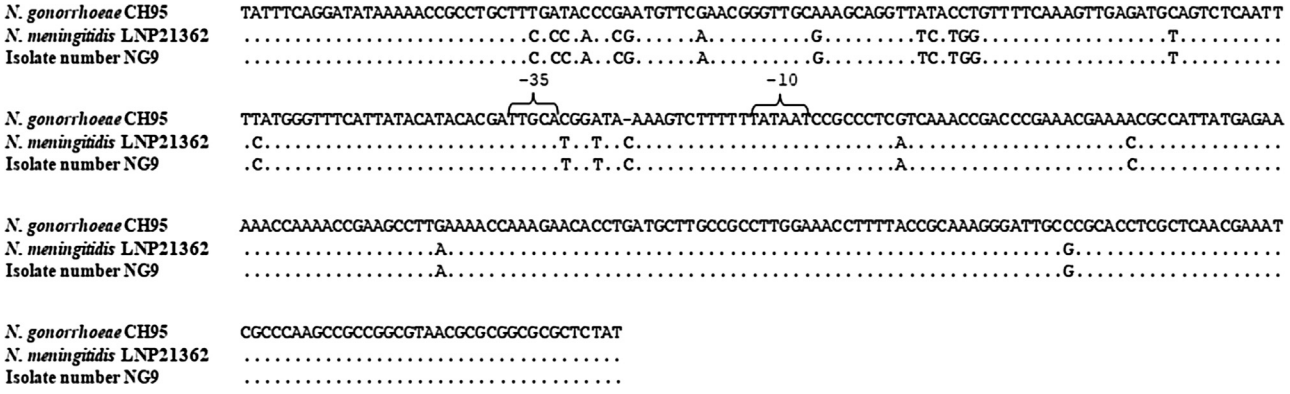

Figure 1. Alignment of partial putative sequences of the *mtrR* promoter from *Neisseria gonorrhoeae* CH95 strain (GenBank accession number Z25796), *Neisseria meningitidis* LNP21362 strain (GenBank accession number CP006869), and an *N. gonorrhoeae* isolate from this study.

Penicillin

In this study, 27% (8/30) of the *N. gonorrhoeae* isolates were susceptible to Pen, 57% (17/30) presented PenI, and 17% (5/30) were PenR, of which 13% (4/30) were classified as PPNG (Table 4). The PBP2 mutation patterns detected in this study were II, V, IX, XII, XIV, XIX, XXII, XXXIX, XXXX, and a PBP2 mosaic XXXIV (Figure 2).

All PenI and PenR isolates studied, with the exception of the PBP2 mosaic isolate, showed an aspartic acid (D) insertion after the 345 position (D345a) in PBP2. In relation to mutations that affect the MtrCDE efflux pump, PenI and PenR isolates showed the following in the MtrR repressor: A39T (4/17), G45D (1/17), A39T/R44H (4/17), and A39T/Y48D (1/17). An adenine deletion (7/17) and a thymine insertion (1/17) were found in the *mtrR* promoter.

Table 4
Minimum inhibitory concentrations (MICs) of PenI and PenR *Neisseria gonorrhoeae* isolates and associated mutations ($n = 18$).

Phenotype	Penicillin MIC (mg/l)	PBP2 mutation pattern[a]	MtrR mutations[b]	*mtrR* promoter mutations	Isolate number
PenI	0.12	II	A39T/R44H	WT	NG18; NG19; NG28
PenI	0.12	II	A39T	WT	NG20; NG26
PenI	0.12	XII	WT	A deletion	NG25
PenI	0.12	XIV	A39T/Y48D	WT	NG13
PenI	0.12	XIX	A39T	WT	NG5
PenI	0.12	XXII	A39T	T insertion	NG11
PenI	0.12	XXXIX	G45D	WT	NG2
PenI	0.12	XXXX	WT	A deletion	NG1
PenI	0.25	II	A39T/R44H	WT	NG16
PenI	0.25	XXXX	WT	A deletion	NG6
PenI	0.25	II	*		NG9
PenI	0.50	IX	WT	A deletion	NG8
PenI	1.00	V	WT	A deletion	NG12
PenI	1.00	XXXIV	WT	A deletion	NG17
PenR	2.00	XXXX	WT	A deletion	NG7

A, adenine; PBP2, penicillin-binding protein 2; PenI, intermediate resistance to penicillin; PenR, resistant to penicillin; T, thymine; WT, wild-type.
[*] *Neisseria meningitidis*-like *mtrR* promoter sequence.
[a] Amino acids 340 to 575 in PBP2.
[b] Amino acids 1 to 60 in MtrR.

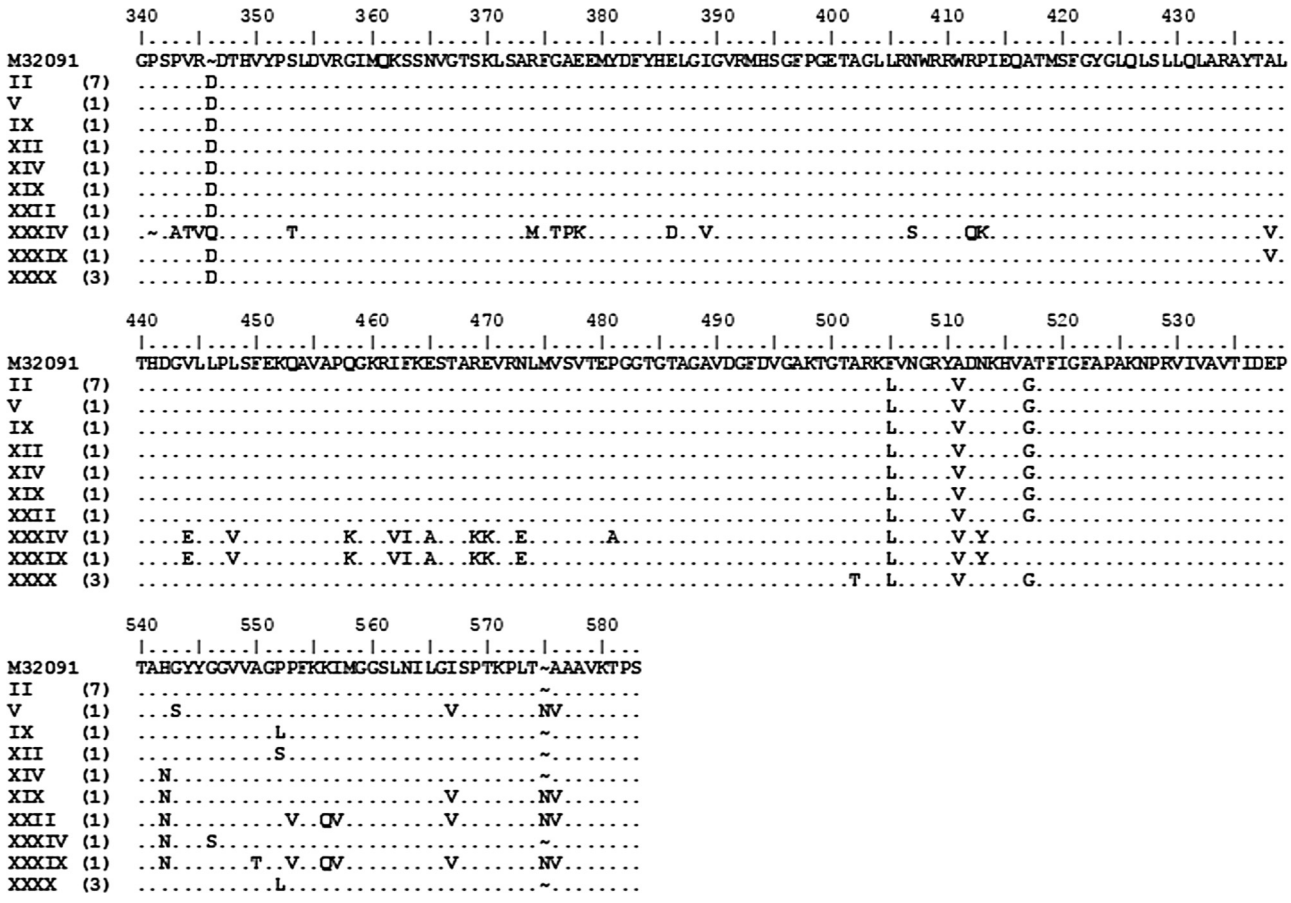

Figure 2. Alignment of the partial putative amino acid sequence of PBP2 from *Neisseria gonorrhoeae* isolates with a sequence M32091. The number of isolates is indicated in parenthesis.

ESCs

All isolates were susceptible to ESCs, except one. This CfmR isolate showed a minimum inhibitory concentration (MIC) of 0.25 mg/l, a PBP2 mosaic XXXIV, and an adenine deletion in the *mtrR* promoter.

Tetracycline

In relation to tetracycline, 53% (16/30) of the isolates were susceptible, 30% (9/30) displayed TetI, and 17% (5/30) were shown to be TetR, of which 6.7% (2/30) were classified as TRNG phenotype (MIC ≥16.0 mg/l (Perilla et al., 2003)). Four in 14 (29%) isolates presented A39T/R44H mutations, two in 14 (14%) presented an A39T mutation, and two in 14 (14%) presented A39T/Y48D mutations in the MtrR. An adenine deletion in the *mtrR* promoter was also detected in another six of 14 (43%) *N. gonorrhoeae* isolates (Table 5).

Ciprofloxacin

In this study, 63% (19/30) of the *N. gonorrhoeae* isolates were susceptible and 37% (11/30) were QRNG isolates. Each isolate presented two mutations in the GyrA subunit (all presented an S91F substitution) and one mutation in the ParC subunit (Table 6).

Azithromycin

Susceptibility to Azm was found in 87% (26/30) of the isolates and intermediate resistance in 13% (4/30). An A39T/R44H double mutation in the MtrR was identified in two Azm-intermediate isolates, while an adenine deletion in the *mtrR* promoter was present in another two.

Spectinomycin

All *N. gonorrhoeae* isolates were susceptible to spectinomycin.

Discussion

Some authors have found *N. gonorrhoeae* isolates with an *N. meningitidis*-like *mtrR* promoter sequence (Trembizki et al., 2014), which was also found in one strain in the present study. The exposure of *Neisseria* spp. to antimicrobial agents (in the treatment of *N. gonorrhoeae* infections or other infections) may result in the selection of resistant strains due to spontaneous genetic mutations and/or due to the acquisition of all or parts of resistance genes (Unemo and Nicholas, 2012).

Penicillin

In this study, the number of PenI isolates was higher than the number of PenR isolates. This situation has also been observed in Canada (Allen et al., 2011), Italy (Starnino et al., 2008), and Russia (Ilina et al., 2008), with 77.1% (115/149), 40.3% (131/326), and 62.2% (289/464) of PenI isolates and 12.1% (18/149), 25.5% (83/326), and 11.9% (55/464) of PenR isolates, respectively. PBP2 patterns have been described by other authors, with sequence patterns I to X (Ito et al., 2005), XI to XXIII (Whiley et al., 2007), and XXXIV to XXXVIII (Allen et al., 2011). Patterns XXXIX and XXXX, which were identified in this study, have already been submitted to GenBank by other authors under accession numbers AKM12408 and WP071198147.1, respectively.

On the other hand, it is also known that the presence of D345a in PBP2 causes penicillin resistance (Fedarovich et al., 2014). Other authors have found this mutation in *N. gonorrhoeae* isolates susceptible to penicillin, probably meaning that additional mutations are necessary in *penA* or other genes (*ponA* and/or *mtrR* and/or *porB*) for the expression of resistance (Ilina et al., 2008). As in the present study, other authors have found mutations A39T, G45D, and A39T/R44H of the MtrR repressor in PenI and PenR isolates (Allen et al., 2011). Regarding the *mtrR* promoter gene, the authors observed an adenine deletion and a thymine insertion in the *mtrR* promoter. According to other authors, these mutations of the MtrCDE efflux pump may contribute to the development of intermediate or full resistance to this antibiotic (Veal et al., 2002; Warner et al., 2008).

ESCs

In a study performed in Portugal, 2.1% (4/187) of isolates obtained between 2004 and 2009 showed decreased susceptibility to Cro (0.125 mg/l ≥ MIC ≤ 0.25 mg/l) (Florindo et al., 2010). In the present study there were no Cro-resistant (CroR) isolates and only 3% were CfmR. No other studies on Cfm or Cro resistance in Portugal are available. However, the European Centre for Disease Prevention and Control (ECDC) have shown that for various reasons, resistance to these two antibiotics has been decreasing since 2010 (ECDC, 2017). The same could be happening in Portugal and that might explain the difference found.

It is also known that PBP2 mosaics confer resistance to ESCs (Ameyama et al., 2002). Only one CfmR isolate that presented a PBP2 mosaic XXXIV was found in the present study. This is worrying, because the *N. gonorrhoeae* F89 strain, which has been identified as extensively resistant to antibiotics (XDR), has shown a high level of CfmR (MIC = 4 mg/l) and CroR (MIC between 1 and 2 mg/l). This strain contains the PBP2 mosaic XXXIV and an additional mutation A501P, which can emerge in strains containing the PBP2 mosaic XXXIV. This may lead to the appearance of XDR strains worldwide, since the presence of *N. gonorrhoeae* isolates with the PBP2 mosaic XXXIV has been reported in several countries (Hess et al., 2012).

Tetracycline

In this study, the number of TetI isolates (30%) was higher than the number of TetR isolates (17%). Studies performed in Canada in

Table 5
Minimum inhibitory concentrations (MICs) present in TetI and TetR *Neisseria gonorrhoeae* isolates and associated mutations ($n = 14$).

Phenotype	Tetracycline MIC (mg/l)	MtrR mutations[a]	*mtrR* promoter mutations	Isolate number
TetI	1	A39T/R44H	WT	NG4; NG14; NG18; NG19
TetI	1	WT	A deletion	NG1; NG6; NG8; NG17
TetI	1	A39T	WT	NG27
TetR	2	WT	A deletion	NG7; NG12
TetR	8	A39T/Y48D	WT	NG29
TRNG	16	A39T/Y48D	WT	NG13
TRNG	32	A39T	WT	NG5

A, adenine; TetI, intermediate resistance to tetracycline; TetR, resistant to tetracycline; TRNG, *N. gonorrhoeae* exhibiting high-level tetracycline resistance; WT, wild-type.
[a] Amino acids 1 to 60 in MtrR.

Table 6
Minimum inhibitory concentrations (MICs) and mutations of QRNG isolates ($n = 11$).

Ciprofloxacin MIC (mg/l)	GyrA mutations[a]	ParC mutations[b]	Isolate number
1	S91F/D95A	S87N	NG26; NG22
1	S91F/D95A	S87R	NG21
2	S91F/D95A	S87N	NG23
4	S91F/D95G	E91G	NG1
4	S91F/D95A	S87N	NG5
4	S91F/D95A	D86N	NG27
8	S91F/D95G	E91G	NG6
8	S91F/D95G	S87R	NG17
>32	S91F/D95G	S87R	NG12
>32	S91F/D95G	E91G	NG7

QRNG, quinolone-resistant *N. gonorrhoeae*.
[a] GyrA amino acids 55 to 110.
[b] ParC amino acids 66 to 119.

2011 (Allen et al., 2011) and Italy in 2008 (Starnino et al., 2008) described 47% (70/149) and 53% (172/326) of TetI isolates and 25.5% (38/149) and 19.1% (62/326) of TetR isolates, respectively. TRNG isolates have been reported by several authors in a higher proportion than that obtained in this study (Cole et al., 2010). Other authors have found similar mutations to those observed in the present study in the TetR and TRNG isolates in the MtrR repressor (Allen et al., 2011). There are other resistance mechanisms that could be involved in the development of resistance to this antibiotic, such as mutations in the *rpsJ* gene and the presence of the *TetM* gene (Unemo and Shafer, 2014).

Ciprofloxacin

The present study findings are similar to those reported in studies from Portugal in 2010 (Florindo et al., 2010) and Italy in 2008 (Starnino et al., 2008), in which 37.4% (70/187) and 34.2% (111/326), respectively, were QRNG isolates. High levels of QRNG have been reported in several European countries, with 63% (861/1366) of isolates (Cole et al., 2011). Several authors have observed that the prevalence of this phenotype remains high in various regions of the world (Unemo and Shafer, 2014). The presence of two mutations in GyrA (including S91F) together with mutations in the ParC protein has been associated with the development of full resistance to ciprofloxacin (Kunz et al., 2012). In this study, all CipR isolates presented these two conditions. The susceptible isolates were not investigated in this study, because other authors have done so and they showed that no isolates that were susceptible to ciprofloxacin had mutations in the *gyrA* or *parC* genes (Giles et al., 2004).

Azithromycin

Isolates with a high level of Azm resistance (AzmR; MIC $\geq$256 mg/l) have been described in different countries (Unemo and Shafer, 2014; Costa-Lourenço et al., 2017). The European Gonococcal Antimicrobial Surveillance Programme reported that AzmR in the MSM population was 8.1% in 2015, a slight decrease from 2014 (10%) (ECDC, 2017). In Portugal, it was reported to be 6.9% in 2006, 8.3% in 2007, and 0% in 2008 and 2009, in accordance with the results of the present study (Cole et al., 2010, 2011).

Mutations in MtrR and in the *mtrR* promoter were found in the present study and have been reported by other authors (Allen et al., 2014). It is known that AzmR in *N. gonorrhoeae* has been associated with the presence of various mutations in the 23S rRNA. It has also been shown to be associated with A39T or G45D mutations in the MtrR repressor or to a single base-pair deletion within the *mtrR* promoter, which affect the expression of the MtrCDE efflux pump (Warner et al., 2008). In relation to this efflux pump, all isolates studied (PenI, PenR, TetI, TetR, CfmR, and AzmI) had at least one of the mutations mentioned above. However, the isolates did not always show resistance to all the pump substrate antibiotics studied. This suggests that an additional resistance mechanism is necessary for resistance to be expressed (Ilina et al., 2008).

Spectinomycin

All *N. gonorrhoeae* isolates in this study were susceptible to Spt. Studies performed in Portuguese individuals (Florindo et al., 2010) and other European individuals (Cole et al., 2010, 2011) have shown similar results.

Conclusions

The recommended first-line regimen for the treatment of *N. gonorrhoeae* in the USA and Europe is the combination of Cro and Azm (Ison et al., 2013). In the present study, circulating *N. gonorrhoeae* isolates in the MSM population of Lisbon were susceptible to Cro, but some isolates presented Azm intermediate resistance and one was resistant to Cfm.

From a global public health perspective, the results of this study are consistent with those of other authors and reinforce the importance of the expedient development of new therapeutic options with new antimicrobial agents or other types of therapeutic molecule.

Taking into account the fact that, as in other studies, some *N. gonorrhoeae* isolates presenting resistance mutations were susceptible to antibiotics, further studies are needed using molecular biology techniques, such as multi-antigen sequence typing, to predict specific antimicrobial resistance.

Acknowledgements

GAT – Portuguese Non-governmental organization (NGO) of people living with HIV, Lisbon, Portugal. Global Health and Tropical Medicine (GHTM) Medical Microbiology Unit, Institute of Hygiene and Tropical Medicine, New University of Lisbon (UNL), Lisbon, Portugal.

Ethical approval

The study was approved by the IHMT Ethics Commission.

Conflict of interest

The authors declare that no conflicts of interest exist.

References

Allen V, Farrell D, Rebbapragada A, Tan J, Tijet N, Perusini S, et al. Molecular analysis of antimicrobial resistance mechanisms in *Neisseria gonorrhoeae* isolates from Ontario, Canada. Antimicrob Agents Chemother 2011;55(2):703–12.
Allen V, Seah C, Martin I, Melano R. Azithromycin resistance is coevolving with reduced susceptibility to cephalosporins in *Neisseria gonorrhoeae* in Ontario, Canada. Antimicrob Agents Chemother 2014;58(5):2528–34.
Ameyama S, Onodera S, Takahata M, Minami S, Maki N, Endo K, et al. Mosaic-like structure of penicillin binding protein 2 gene (penA) in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. Antimicrob Agents Chemother 2002;46(12):3744–9.
Cole M, Chisholm S, Hoffmann S, Stary A, Lowndes C, Ison C. European surveillance of antimicrobial resistance in *Neisseria gonorrhoeae*. Sex Transm Infect 2010;86 (6):427–32.
Cole M, Unemo M, Hoffmann S, Chisholm S, Ison C, Laar M. The European gonococcal antimicrobial surveillance programme, 2009. Euro Surveill 2011;16(42):1–6.
Costa-Lourenço A, Santos K, Moreira B, Fracalanzza S, Bonelli R. Antimicrobial resistance in *Neisseria gonorrhoeae*: history, molecular mechanisms and epidemiological aspects of an emerging global threat. Braz J Microbiol 2017;48(4):617–28.
Cousin S, Whittington W, Roberts M. Acquired macrolide resistance genes in pathogenic Neisseria spp. isolated between 1940 and 1987. Antimicrob Agents Chemother 2003;47(12):3877–80.

ECDC. Annual epidemiological report: reportingon 2011 surveillance data and 2012 epidemic intelligence data. Stockholm: ECDC; 2013.
ECDC. Gonococcal antimicrobial susceptibility surveillance in Europe 2015. Stockholm: ECDC; 2017.
EUCAST. Breakpoint tables for interpretation of MICs and zone diameters: version 4.0. 2014. Available from: http://www.eucast.org. [cited 2014 November 15].
Fedarovich A, Cook E, Tomberg J, Nicholas RA, Davies C. Structural effect of the Asp345a insertion in penicillin-binding protein 2 from penicillin-resistant strains of *Neisseria gonorrhoeae*. Biochem 2014;53(48):7596–903.
Florindo C, Pereira R, Boura M, Nunes B, Paulino A, Gomes J, et al. Genotypes and antimicrobial-resistant phenotypes of *Neisseria gonorrhoeae* in Portugal (2004-2009). Sex Transm Infect 2010;86(6):449–53.
Giles J, Falconio J, Yuenger J, Zenilman J, Dan M, Bash M. Quinolone resistance-determining region mutations and por type of *Neisseria gonorrhoeae* isolates: resistance surveillance and typing by molecular methodologies. J Infect Dis 2004;189(11):2085–93.
Hess D, Wu A, Golparian D, Esmaili S, Pandori W, Sena E, et al. Genome sequencing of a *Neisseria gonorrhoeae* isolate of a successful international clone with decreased susceptibility and resistance to extended-spectrum cephalosporins. Antimicrob Agents Chemother 2012;56(November (11)):5633–41.
Ilina E, Vereshchagin V, Borovskaya A, Malakhova M, Sidorenko S, Al-Khafaji N, et al. Relation between genetic markers of drug resistance and susceptibility profile of clinical *Neisseria gonorrhoeae* strains. Antimicrob Agents Chemother 2008;52(6):2175–82.
Ison C, Golparian D, Saunders P, Chisholm S, Unemo M. Evolution of *Neisseria gonorrhoeae* is a continuing challenge for molecular detection of gonorrhoea: false negative gonococcal porA mutants are spreading internationally. Sex Transm Infect 2013;89(3):197–201.
Ito M, Deguchi T, Mizutani K-S, Yasuda M, Yokoi S, Ito S-I, et al. Emergence and spread of *Neisseria gonorrhoeae* clinical isolates harboring mosaic-like structure of penicillin binding protein 2 in central Japan. Antimicrob Agents Chemother 2005;49(1):137–43.
Kunz A, Begum A, Wu H, D'Ambrozio J, Robinson J, Shafer W, et al. Impact of fluoroquinolone resistance mutations on gonococcal fitness and in vivo selection for compensatory mutations. J Infect Dis 2012;205(12):1821–9.
Liao M, Gu W-M, Yang Y, Dillon J-A. Analysis of mutations in multiple loci of *Neisseria gonorrhoeae* isolates reveals effects of PIB, PBP2 and MtrR on reduced susceptibility to ceftriaxone. J Antimicrob Chemother 2011;66(5):1016–23.
Perilla M, Ajello G, Bopp C, Elliott J, Facklam R, Knapp J, et al. Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world. Atlanta: WHO/CDC; 2003.
Starnino S, Suligoi B, Regine V, Bilek N, Stefanelli P, Dal Conte I, et al. Phenotypic and genotypic characterization of *Neisseria gonorrhoeae* in parts of Italy: detection of a multiresistant cluster circulating in a heterosexual network. Clin Microbiol Infect 2008;14:949–54.
Tanaka M, Nakayama H, Haraoka M, Saika T, Kobayashi I, Naito S. Antimicrobial resistance of *Neisseria gonorrhoeae* and high prevalence of ciprofloxacin-resistant isolates in Japan, 1993 to 1998. J Clin Microbiol 2000;38(2):521–5.
Tapsall J, Ndowa F, Lewis D, Unemo M. Meeting the public health challenge of multidrug and extensively drug resistant *Neisseria gonorrhoeae*. Expert Rev Anti Infect Ther 2009;7(7):821–34.
Trembizki E, Doyle C, Jennison A, Smith H, Bates J, Lahra M, et al. A *Neisseria gonorrhoeae* strain with a meningococcal mtrR sequence. J Med Microbiol 2014;63:1113–5.
Unemo M, Ballard R, Ison C, Lewis D, Ndowa F, Peeling R. In: Unemo M, Ballard R, Ison C, Lewis D, Ndowa F, Peeling R, editors. Laboratory diagnosis of sexually transmitted infections, including human immunodeficiency virus. Geneva: WHO; 2013.
Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. Futur Microbiol 2012;7(12):1401–22.
Unemo M, Shafer W. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st Century: past, evolution, and future. Clin Microbiol Rev 2014;27(3):587–613.
Veal W, Nicholas R, Shafer W. Overexpression of the MtrC-MtrD-MtrE efflux pump due to an mtrR mutation is required for chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. J Bacteriol 2002;184(20):5619–24.
Warner D, Shafer W, Jerse A. Clinically relevant mutations that cause derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol Microbiol 2008;70(2):462–78.
Whiley D, Limnios E, Ray S, Sloots T, Tapsall J. Diversity of penA alterations and subtypes in *Neisseria gonorrhoeae* strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob Agents Chemother 2007;51(9):3111–6.