Epidemiological characteristics and antibiotic susceptibility of *Neisseria gonorrhoeae* isolates from Greece during 2009–2023

Eirini Siatravani,[1,2] Anna-Danai Panopoulou,[3] Michalis Polemis,[4] Stella-Eugenia Chryssou,[3] Ioannis Moschos,[5] Ioanna Magaziotou,[6] Apostolos Beloukas ,[2] Eva Tzelepi,[1] Vivi Miriagou [1]

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[1]National Reference Center for Neisseria gonorrhoeae, Laboratory of Bacteriology, Hellenic Pasteur Institute, Athens, Greece
[2]Department of Biomedical Sciences, University of West Attica, Egaleo, Athens, Greece
[3]Laboratory of Biopathology, 'Andreas Syggros' Hospital of Cutaneous and Venereal Diseases, Athens, Greece
[4]Computational Biology Department, Central Public Health Laboratory, National Public Health Organization, Vari, Athens, Greece
[5]Microbiology Laboratory, Venereal and Skin Diseases Hospital of Thessaloniki, Thessaloniki, Greece
[6]Department for Sexually Transmitted Infections, National Public Health Organization, Athens, Greece

Correspondence to
Dr Vivi Miriagou; miriagou@pasteur.gr

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ABSTRACT

Objectives To monitor epidemiological characteristics and antibiotic susceptibility trends of *Neisseria gonorrhoeae* in Greece during 2009–2023.

Methods Microbiological and epidemiological data for 1756 gonococci received by the Greek National Reference Centre for *Neisseria gonorrhoeae* were evaluated. Strains were isolated consecutively from gonorrhoea cases in hospitals throughout Greece. Minimum inhibitory concetrations of antibiotics were determined by E-test. Plasmid content analysis was performed for penicillinase-producing isolates (PPNG) and for isolates exhibiting tetracycline resistance (TRNG). *tetM*, *penA, gyrA* and *parC* genes were identified by PCR and RFLP/sequencing. Isolates were subjected to serotyping. Genomic analysis by pulsed-field gel electrophoresis (PFGE) was performed for extended-spectrum cephalosporin (ESC)-resistant isolates.

Results Only 2.8% of the isolates were fully susceptible to all antibiotics. High rates of resistance were observed for penicillin G (27.5%), tetracycline (59.2%) and ciprofloxacin (68.8%). PPNG and/or TRNG isolates accounted for 26% of the total sample, the majority (81.6%) being simultaneously quinolone-resistant (QRNG). The isolation frequency of QRNG isolates was stably high as in previous years. Interestingly, a proportion of QRNG isolates exhibited cross-resistance to all antibiotics except spectinomycin. Azithromycin resistance is showing an increasing trend since 2021 at alarming levels (32.7% in 2023). The percentage of isolates exhibiting decreased susceptibility to ESCs (CDS) remained stable until 2019, whereas no CDS strains were isolated from 2020 to 2023. Spectinomycin was active against all isolates. Serotyping results revealed a strong association of quinolone resistance with Bpyut, Bpyust and Bropyst serovars and I/S phenotypes with Bpyvut and Byut serovars. PFGE showed that CDS isolates were classified into eight groups, with the majority clustered in three main clones including the predominant CDS clone isolated during 2001–2008.

Conclusions The gonococcal population showed a continuous change in the resistance phenotypes and predominating clones during 2009–2023 underlining the need for continuous monitoring of the traits of this pathogen.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* (NG) is a dynamic and evolving challenge.
⇒ Resistance patterns of NG to various antibiotic groups are constantly changing.

WHAT THIS STUDY ADDS

⇒ This study provides data on NG-AMR in Greece during a 15-year study period (2009–2023).
⇒ Resistance profiles of the studied gonococcal population show that quinolone resistance remains consistently at high levels and is the predominant resistance phenotype, either alone or in combination with other resistance markers.
⇒ The changing resistance patterns of NG during different periods were apparently due to selection and circulation of diverse NG clones in the Greek community.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study emphasises the need for continuous monitoring of resistance trends in European communities.
⇒ Meticulous following up of NG epidemiology and resistance trends are crucial for the implementation of Public Health Policies and adaptation of antibiotic treatment.
⇒ Based on surveillance data for antimicrobial susceptibility of the gonococcal population in Greece, the empirical treatment of patients with gonorrhoea is guided at the national level.

INTRODUCTION

Neisseria gonorrhoeae (NG) is the causative agent of gonorrhoea, which, after chlamydia infections, is the second most common sexually transmitted disease (STD) of bacterial aetiology worldwide.[1] According to WHO, gonorrhoea mainly affects certain key population groups such as people with promiscuous sexual behaviour, men having sex with men (MSM), people working in the sex industry, transgender women and adolescents and young people in countries with a high disease burden. Gonococcus is inherently sensitive to antibiotics but can develop resistance to various groups of

antibiotics either by chromosomal mutations or acquisition of resistance determinants.[2 3]

The characteristics of patients with gonorrhoea prior to 2009 showed: (a) a stable low incidence of recorded gonorrhoea cases during the 2001–2008 period (range: one to two cases per 100 000 population), (b) the majority of patients with gonorrhoea were aged 20–40 years (annual range: 69%–81.5%) and (c) the percentage of MSM with gonorrhoea ranged from 21.3% to 36.2%.[4]

The antimicrobial resistance in NG population in this period exhibited fluctuations. From 2001 to 2008, plasmid-mediated resistance to penicillin and tetracycline among gonococci was seen at relatively low frequencies.[5 6] On the other hand, a steep increase in resistance to fluoroquinolones was noted since 2004.[5–7] Moreover, between 2006 and 2008, the spread of a clone with decreased susceptibility to third-generation cephalosporins (CDS) was documented.[8] It is evident that close monitoring of the gonococcal population dynamics as well as the antibiotic resistance trends are essential for implementing infection control policies and guiding empirical therapy.

In this study, we present data from the epidemiological surveillance conducted during 2009–2023 regarding the epidemiological characteristics of gonococcal infections, as well as the antibiotic resistance patterns and genotypes of gonococci isolated in Greece during the aforementioned 15-year period.

MATERIALS AND METHODS

Neisseria gonorrhoeae isolates

A total of 1756 gonococcal isolates collected during January 2009–December 2023 were studied. Of these, 1729 (98.5%) were from males, 18 from females and nine of unknown gender. Most gonococci (n=1408; 80.2%) were consecutively isolated in the outpatient clinic of the 'Andreas Syggros' STD Hospital, Athens. Twenty-four strains (1.4%) were isolated in the Venereal and Skin Diseases Hospital of Thessaloniki during 2009–2012. The remaining 324 isolates were from sporadic cases encountered in general hospitals located in Central and South Greece. This collection represented the total of the viable non-repetitive isolates obtained from all collaborating hospitals.

Epidemiological data

Epidemiological data were obtained from the patients during hospital visits by answering a standardised questionnaire regarding age, nationality, place of residence, recent travel, sexual orientation, previous history of STDs and relevant information about the partner(s) in suspected infectious contacts. The data forwarded to the Greek National Reference Centre for *Neisseria gonorrhoeae* were pseudonymised.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities were determined for penicillin G, tetracycline, azithromycin, ciprofloxacin, cefotaxime, ceftriaxone, cefixime and spectinomycin as recommended by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucast.org/clinical_breakpoints). Cefotaxime was tested for the years 2009–2019. Azithromycin was tested for the years 2011–2023. Azithromycin ECOFF value of minimum inhibitory concetration (MIC) >1 mg/L was retrospectively used as the resistance breakpoint. Revised tetracycline-resistant breakpoints (EUCAST V.13, 1 January 2023) were used for all isolates. MICs were determined by the E-test method (AB Biodisk, Biomerieux) as described previously.[6] The NGstrains WHO A, B, C and D were used for quality control purposes. Penicillinase-producing *N. gonorrhoeae* (PPNG) isolates were detected using a nitrocefin assay.

Resistance genes

Genomic DNA was purified from bacterial cultures using the Quick-gDNA miniprep kit (ZymoResearch).

tetM

The presence of *tetM* genes of plasmid origin in highly tetracycline-resistant isolates (TRNGs) was confirmed by PCR amplification. Additional characterisation of the *tetM* gene type was done by RFLP analysis using the MspI restriction enzyme.[9]

penA

penA genes were identified in the genomic DNA of representative isolates exhibiting CDS by PCR amplification using specific primers that included the entire gene.[10] Nucleotide sequences of the PCR products were determined by Sanger sequencing.

gyrA and *parC* quinolone resistance-determining regions (QRDR)

In QRNG isolates, mutations in the *gyrA* and *parC* genes were identified by PCR and sequencing of the QRDR regions.[11 12]

Resistance plasmids

Plasmid content analysis of PPNG and TRNG isolates was carried out by plasmid DNA extraction and electrophoresis on agarose gels.[13]

Typing of the isolates

Serotyping

Serotyping of gonococcal isolates was performed using monoclonal antibodies (MAbs) recognising individual epitopes on the major protein I (Por I) of the bacterial outer membrane. Serological determination was performed using the Phadebact GC serovar panels IA and IB (MKL Diagnostics). Serotyping was performed for all isolates isolated during 2009–2022.

PFGE typing of selected isolates

Isolates were typed by pulsed-field gel electrophoresis (PFGE). Genomic DNA analysis after DNA fragmentation with BcuI was performed as previously described.[14] Fingerprints were analysed using the Gel Compar II V.4.1 software (Applied Maths). The percentage of similarity was calculated with the Dice coefficient (position tolerance 2.5%). A pattern similarity of >80% was considered as indicating genetic relatedness. PFGE was applied to 154 representative isolates exhibiting decreased susceptibility to at least one of the ESCs tested (ceftriaxone, cefixime or cefotaxime).

Nucleotide sequence accession numbers

Nucleotide sequences of *penA* genes of representative isolates A3165 and A3210 have been assigned GenBank accession numbers KU321636 and KU321637.

RESULTS

Epidemiological data

The incidence of recorded gonorrhoea cases during the 2009–2023 period remained stably low (range 1.16–2.19 cases per 100 000 population) reaching the highest incidence in 2023 (online supplemental figure S1). The mean age of

adult male patients (n=1728) was 35 years (age range 14–80 years). For adult female patients (n=16), the mean age was 41 years (range 18–74 years). There were also two cases of gonorrhoea in underage girls and a case of eye infection in a boy aged 3 years. Gender for nine cases was unknown. In total, 1610 (91.7%) isolates originated from the urethra, nine (0.5%) from the cervix and 10 (0.6%) from the anus. A small number of isolates were derived from semen, urine, eye, vagina and pharynx (n=24). For the remaining isolates (n=103, 5.9%), information regarding the site of infection was not available. The patients were scattered into 45 nationalities, most of them being native Greeks (n=1304, 74.3%). The remaining nationalities made up 18.7% (n=328), while a small percentage (n=124, 7.1%) was of unknown ethnicity. The variety of nationalities most likely reflects the migration flows occurring in Greece during the study. The percentage of patients from Balkan countries and Turkey was 8.6% (n=152). The remaining patients were from South, Eastern and Central Europe (n=42, 2.4%), Africa (n=38, 2.2%), Asia (n=48, 2.7%) and Middle East (n=39, 2.2%). A small number of patients (n=9, 0.51%) originated from North and South America, Australia and Western Europe.

Regarding the sexual orientation of the patients, the majority were heterosexual (n=974, 55.5%) followed by MSM-homosexual (n=386, 22.0%) and MSM-bisexual (n=140, 7.9%) patients. The percentage of MSM patients showed an increasing trend during 2009–2023, with the lowest value in 2011 (17.3%) and the highest in 2022 (46.5%). For the remaining 256 cases (14.6%), sexual orientation was not recorded.

Antimicrobial susceptibility and mechanisms of resistance

Antibiotic susceptibilities are presented in table 1. Fifty-two isolates were susceptible to all antibiotics (2.8%). There was a progressive decrease in the number of isolates without resistance characters in gonococci isolated in Greece during 2009–2023 as compared with previous periods.[5 6] Spectinomycin continues to exhibit 100% susceptibility as in previous years.[5–7] Figure 1A,B show the resistance rates of antibiotics tested and the resistance phenotypes.

Penicillin and tetracycline resistance

Plasmid-mediated resistance to penicillin and tetracycline

Isolates with PPNG and TRNG were isolated at frequencies of 11.8% (n=207) and 14.2% (n=249), respectively (table 1). Notably, 7.3% of isolates (n=128) possessed both penicillin and tetracycline resistance plasmids. The MICs of penicillin ranged from 3 to >256 mg/L and of tetracycline from 2 to 256 mg/L for PPNG and TRNG isolates, respectively. In total, 18.6% of isolates (326 out of 1756) showed plasmid-mediated resistance to penicillin and/or tetracycline (PMR). Most of the PMR isolates (n=266 out of the 326, 81.6%) also exhibited co-resistance to fluoroquinolones (QR-PMR) (figure 1B). The predominant plasmid in the PPNG isolates was of the African type (3.2 MDa)[15 16] identified in 186 out of 207 PPNG isolates. The Asian plasmid (4.5 MDa)[15 16] was detected in only four PPNG isolates. The remaining PPNG isolates (n=17) had a plasmid of 3.05 MDa corresponding to the Toronto type.[16] Similarly, for TRNG isolates, the predominant resistance plasmid (25.2 MDa) carried the Dutch type *tetM* gene and was identified in 171 out of the 249 TRNG isolates. Seventy-five isolates harboured the American type and three had the Uruguayan type *tetM* gene.[16]

Chromosomally mediated low resistance level

Of the 1756 isolates, 738 (35.2%) showed chromosomal resistance (cmR) to one or more antibiotics (penicillin, tetracycline) (figure 1B). Specifically, 275 isolates (15.7%) were chromosomally resistant to penicillin (MIC range 1.5–16 mg/L, non-PPNG) (CMPR) and 790 (45.0%) to tetracycline (MIC range 0.75–6 mg/L, non-TRNG) (CMTR) (table 1). During the 15-year period, variations were observed for penicillin and tetracycline resistance rates, fluctuating from 1.0% to 46.5% and 23.6% to 71.7%, respectively, with a peak for both antibiotics in 2013. However, an overall decreasing trend was observed for both penicillin and tetracycline resistance rates (figure 1A).

Resistance to fluoroquinolones

Of the 1756 isolates tested, 1208 (68.8%) were resistant to fluoroquinolones (MIC range: 0.094–>32 mg/L) (table 1). Notably, 281 isolates (16.0%) were found to be solely resistant to quinolones (QR), whereas the remaining 927 isolates were multiresistant. Of the 1208 quinolone-resistant isolates, 266 also exhibited plasmid-mediated resistance to penicillin and/or tetracycline (15.1%) (QR-PMR). The percentage of isolates with resistance to quinolones and chromosomal cross-resistance to one or more antibiotics (QR-cmR) was 21.5% (n=378). Two hundred thirty-three isolates (13.3%) showed resistance to quinolones combined with resistance to ESC and chromosomal resistance to one or more antibiotics (QR-CDS). The QRDR regions of *gyrA* and *parC* genes of representative isolates were analysed. Mutations conferring resistance to quinolones were mapped in the *gyrA* gene in all isolates, resulting in the amino acid substitutions Val81Ile, His86Leu, Ser91Phe, Ala92Pro and Asp95Gly or Asp95Ala. The *parC* gene mutations included Asp86Asn, Ser87Arg or Ser87Asn or Ser87Ile, Ser88Pro and Glu91Gly alterations.

Macrolide resistance

Among the 1466 isolates tested for azithromycin susceptibility during the period 2011–2023, 125 (8.5%) azithromycin-resistant isolates (ARNG) with MIC ranging from 1.5 to >256 mg/L were isolated (table 1). During the 2009–2020 period, ARNG isolates appeared sporadically; however, since 2021, a significant increase in azithromycin resistance has been observed, with a peak of 32.7% in 2023. An overall increasing trend is observed for azithromycin resistance (figure 1A). High-level resistance to azithromycin was not observed up to 2022, but in 2023 isolates with MIC ≥256 mg/L were isolated (n=6).

Resistance to third-generation cephalosporins

Of the 1756 isolates, the resistance rates for ceftriaxone and cefixime were 0.4% (n=7) and 6.1% (n=107). The resistance rate during 2009–2019 for cefotaxime was 20.9% (n=265) (table 1). The percentage of CDS strains remained stable compared with 2005–2008 (7.9%).[7] Notably, after 2016, a decreasing trend in the resistance rate was observed, and since 2021, no CDS strains were isolated. All CDS strains were multidrug resistant, with cross-resistance to the newer quinolones and chromosomal-type resistance to other antibiotic classes (figure 1B). Cephalosporin marginally susceptible (CMS) strains, showing MIC 0.094–0.125 mg/L to at least one of the three cephalosporins tested, were also studied. The total number of CMS isolates was 164 (9.3%) (ceftriaxone 106 (6.0%) and cefixime 58 (3.3%)).

Table 1 Antimicrobial susceptibilities of 1756 *Neisseria gonnorrhoeae* isolates isolated in Greece during 2009–2023

Antimicrobial agent	Susceptibility category	MIC breakpoints (mg/L)	MIC range (mg/L)	2009 n=140 (%)	2010 n=150 (%)	2011 n=144 (%)	2012 n=70 (%)	2013 n=99 (%)	2014 n=111 (%)	2015 n=127 (%)	2016 n=157 (%)
Penicillin G	PPNG	Pen/ase production	3–>256	9 (6.4)	9 (6.0)	13 (9.0)	8 (11.4)	4 (4.0)	6 (5.4)	14 (11.0)	42 (26.7)
	CMPR	>1 (non-PPNG)	1.5–16	27 (19.3)	34 (22.7)	39 (27.1)	13 (18.6)	46 (46.5)	44 (39.6)	25 (19.7)	15 (9.6)
	Intermediate	0.094–1	0.094–1	87 (62.1)	98 (65.3)	80 (55.6)	42 (60.0)	48 (48.5)	60 (54.1)	87 (68.5)	100 (63.7)
	Susceptible	≤0.064	≤0.016–0.064	17 (12.1)	9 (6.0)	12 (8.3)	7 (10.0)	1 (1.0)	1 (0.9)	1 (0.8)	0 (0.0)
Tetracycline	TRNG	Positive tetM-PCR	2–256	21 (15.0)	33 (22.0)	27 (18.8)	15 (21.4)	9 (9.1)	2 (1.8)	7 (5.5)	30 (19.1)
	CMTR	>0.5 (non-TRNG)	0.75–6	66 (47.1)	75 (50.0)	72 (50.0)	33 (47.1)	71 (71.7)	77 (69.3)	88 (69.3)	74 (47.1)
	Susceptible	≤0.5	0.016–0.5	53 (37.9)	42 (28.0)	45 (31.3)	22 (31.4)	19 (19.2)	32 (28.8)	32 (25.2)	53 (33.7)
Ciprofloxacin	Resistant	>0.06	0.094–>32	93 (66.4)	108 (72.0)	113 (78.5)	50 (71.4)	70 (70.7)	77 (69.4)	99 (78.0)	101 (64.3)
	Intermediate	0.06	0.064	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Susceptible	≤0.03	0.002–0.047	47 (33.6)	42 (28.0)	31 (21.5)	20 (28.6)	28 (28.3)	34 (30.6)	28 (22.0)	56 (35.7)
Azithromycin*	Resistant	>1	1.5–>256	NT	NT	0 (0.0)	1 (1.4)	1 (1.0)	5 (4.5)	1 (0.8)	8 (5.1)
	Susceptible	≤1	0.016–1	NT	NT	144 (100.0)	69 (98.6)	98 (99.0)	106 (95.5)	126 (99.2)	149 (94.9)
Ceftriaxone	Resistant	>0.125	0.190–0.5	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (3.6)	2 (1.6)	0 (0.0)
	Susceptible	≤0.125	0.016–0.125	139 (99.3)	150 (100.0)	144 (100.0)	70 (100.0)	99 (100.0)	107 (96.4)	125 (98.4)	157 (100.0)
Cefixime	Resistant	>0.125	0.190–0.750	19 (13.6)	10 (6.7)	12 (8.3)	6 (8.6)	20 (20.2)	12 (10.8)	11 (8.7)	5 (3.2)
	Susceptible	≤0.125	0.016–0.125	121 (86.4)	140 (93.3)	132 (91.7)	64 (91.4)	79 (79.8)	99 (89.2)	116 (91.3)	152 (96.8)
Cefotaxime†	Resistant	>0125	0.190–2	22 (15.7)	43 (28.7)	46 (32.0)	17(24,2)	34 (34.3)	38 (34.2)	30 (23.6)	15 (9.5)
	Susceptible	≤0.125	0.016–0.125	118 (84.3)	107 (71.3)	98 (68.0)	53 (75.8)	65 (65.7)	73 (65.8)	97 (76.4)	142 (90.4)
Spectinomycin	Resistant	>64	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Susceptible	≤64	≤48	140 (100.0)	150 (100.0)	144 (100.0)	70 (100.0)	99 (100.0)	111 (100.0)	127 (100.0)	157 (100.0)

Antimicrobial Agent	Susceptibility Category	MIC Breakpoints (mg/L)	MIC Range (mg/L)	2017 n=89 (%)	2018 n=83 (%)	2019 n=99 (%)	2020 n=114 (%)	2021 n=107 (%)	2022 n=101 (%)	2023 n=165 (%)	Total n=1756 (%)
	PPNG	Pen/ase production	3->256	13 (14.6)	12 (14.5)	23 (23.2)	30 (26.3)	5 (4.7)	9 (8.9)	10 (6.1)	207 (11.8)
Penicillin G	CMPR	>1 (non-PPNG)	1.5–16	12 (13.5)	4 (4.8)	4 (4.0)	2 (1.8)	6 (5.6)	1 (1.0)	3 (1.8)	275 (15.7)
	Intermediate	0.094-1	0.094-1	52 (58.4)	56 (67.5)	68 (68.7)	74 (64.9)	95 (88.8)	84 (83.2)	140 (84.8)	1171 (66.7)
	Susceptible	≤0.064	≤0.016-0.064	12 (13.5)	11 (13.2)	4 (4.0)	8 (7.0)	1 (0.9)	7 (6.9)	12 (7.3)	103 (5.8)
Tetracycline	TRNG	Positive tetM-PCR	≤0.016–0.064	11 (12.4)	12 (14.5)	33 (33.3)	28 (24.5)	7 (6.5)	4 (4.0)	10 (6.1)	249 (14.2)
	CMTR	>0.5 (non-TRNG)	2–256	21 (23.6)	31 (37.3)	24 (24.2)	50 (43.9)	32 (30.0)	30 (29.7)	46 (27.9)	790 (45.0)
	Susceptible	≤0.5	0.75–6	57 (64.0)	40 (48.2)	42 (42.4)	36 (31.6)	68 (63.5)	67 (66.3)	109 (66.1)	717 (40.8)
Ciprofloxacin	Resistant	>0.06	0.094–>32	49 (55.1)	47 (56.6)	59 (59.6)	98 (86.0)	74 (69.2)	67 (66.3)	103 (62.4)	1208 (68.8)
	Intermediate	0.06	0.064	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.06)
	Susceptible	≤0.03	0.002–0.047	40 (44.9)	36 (43.4)	40 (40.4)	16 (14.0)	33 (30.8)	34 (33.7)	62 (37.6)	547 (31.1)
Azithromycin*	Resistant	>1	1.5–>256	2 (2.2)	1 (1.2)	4 (4.0)	3 (2.6)	20 (18.7)	25 (24.8)	54 (32.7)	125 (8.5)
	Susceptible	≤1	0.016–1	87 (97.8)	82 (98.8)	95 (96.0)	111 (97.4)	87 (81.3)	76 (75.2)	111 (67,3)	1341 (91.5)
Ceftriaxone	Resistant	>0.125	0.190–0.5	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (0.4)
	Susceptible	≤0.125	0.016–0.125	89 (100.0)	83 (100.0)	99 (100.0)	114 (100.0)	107 (100.0)	101 (100.0)	165 (100.0)	1749 (99.6)

Continued

Table 1 Continued

Antimicrobial Agent	Susceptibility Category	MIC Breakpoints (mg/L)	MIC Range (mg/L)	2017 n=89 (%)	2018 n=83 (%)	2019 n=99 (%)	2020 n=114 (%)	2021 n=107 (%)	2022 n=101 (%)	2023 n=165 (%)	Total n=1756 (%)
Cefixime	Resistant	>0.125	0.190–0.750	5 (5.6)	5 (6.0)	1 (1.0)	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	107 (6.1)
	Susceptible	≤0.125	0.016–0.125	84 (94.4)	78 (94.0)	98 (99.0)	113 (99.1)	107 (100.0	101 (100.0)	165 (100.0)	1649 (93.9)
Cefotaximeŧ	Resistant	>0,125	0.190–2	7 (7.9)	8 (9.6)	5 (5.1)	NT	NT	NT	NT	265 (20.9)
	Susceptible	≤0.125	0.016–0.125	82 (92.1)	75 (90.4)	94 (94.9)	NT	NT	NT	NT	1004 (79.1)
Spectinomycin	Resistant	>64	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Susceptible	≤64	≤48	89 (100.0)	83 (100.0)	99 (100.0)	114 (100.0)	107 (100.0)	101 (100.0)	165 (100.0)	1756 (100.0)

*1466 isolates were tested for azithromycin.
†1269 isolates were tested for cefotaxime.
.CMPR, chromosomally resistant to penicillin; CMTR, chromosomally resistant to tetracycline; MIC, minimum inhibitory concetration; NT, not tested; PPNG, penicillinase-producing *N. gonorrhoeae*; TRNG, highly resistant to tetracycline.

Based on the antibiotic susceptibility patterns, 20 representative isolates (16 CDS and four CMS) were selected for analysis of their *penA* gene. All CDS isolates tested carried the same *penA* gene coding for a mosaic PBP-2 transpeptidase with 100% identity to the one deposited under GenBank accession no. HQ204560 sequence.[17] The four CMS isolates had *penA* genes encoding non-mosaic PBP-2 proteins with the characteristic presence of an additional aspartic acid at amino acid position 345 of the protein sequence (Asp345a insertion), exhibiting 99% homology to the GenBank accession no. X07470.[18]

Strain types and their association with antibiotic resistance mechanisms

The 1756 isolates exhibited significant serotypic diversity, being distributed in 101 serotypes. In particular, 88 serotypes of the IB and 13 serotypes of the IA serogroups were identified; Bpyut (16.5%) followed by Bpyust, Bpyvut, Bpyst, Bropyst, Byut and Bropt serotypes and Ast (2.6%) followed by Arst and Ars serotypes were the most prevalent in IB and IA serogroups, respectively. Serotyping results are included in figure 2, where the distribution of isolates in the most prevalent serotypes is depicted. Antibiotic resistance phenotypes for each serotype are shown in the same histogram. Ast, Arst and Ars serotypes were linked to quinolone-resistant strains QR or QR-PMR. Bpyvut and Byut serotypes were mainly associated with isolates exhibiting intermediate susceptibility (I/S) phenotypes. Bpyut, Bpyust and Bropyst serotypes were strongly associated with QR, QR-cmR, QR-PMR and QR-CDS phenotypes. Of note, the Bpyut serovar was predominant among the ESC-resistant gonococcal isolates in Northern Greece in the previous years, 2006–2008.[8]

All CDS and CMS strains (period 2009–2019), belonging to different serotypes, were subjected to PFGE typing. Selected isolates from previous years with characteristic PFGE patterns were also included in the PFGE for comparison. PFGE typing classified the isolates in eight different groups (A, B, C, D, E, F, G and H) and three unique pulsotypes (U1, U2, U3). The majority of the isolates were classified as A, C and D. Type C is one of the CDS pulsotypes that predominated among CDS clones in the previous time period (represented by isolate T6124_2007; figure 3).[6] The CMS isolates were distributed into subtypes of the main pulsotypes A, C and F. Figure 3 shows the PFGE profiles of representative CDS and CMS isolates.

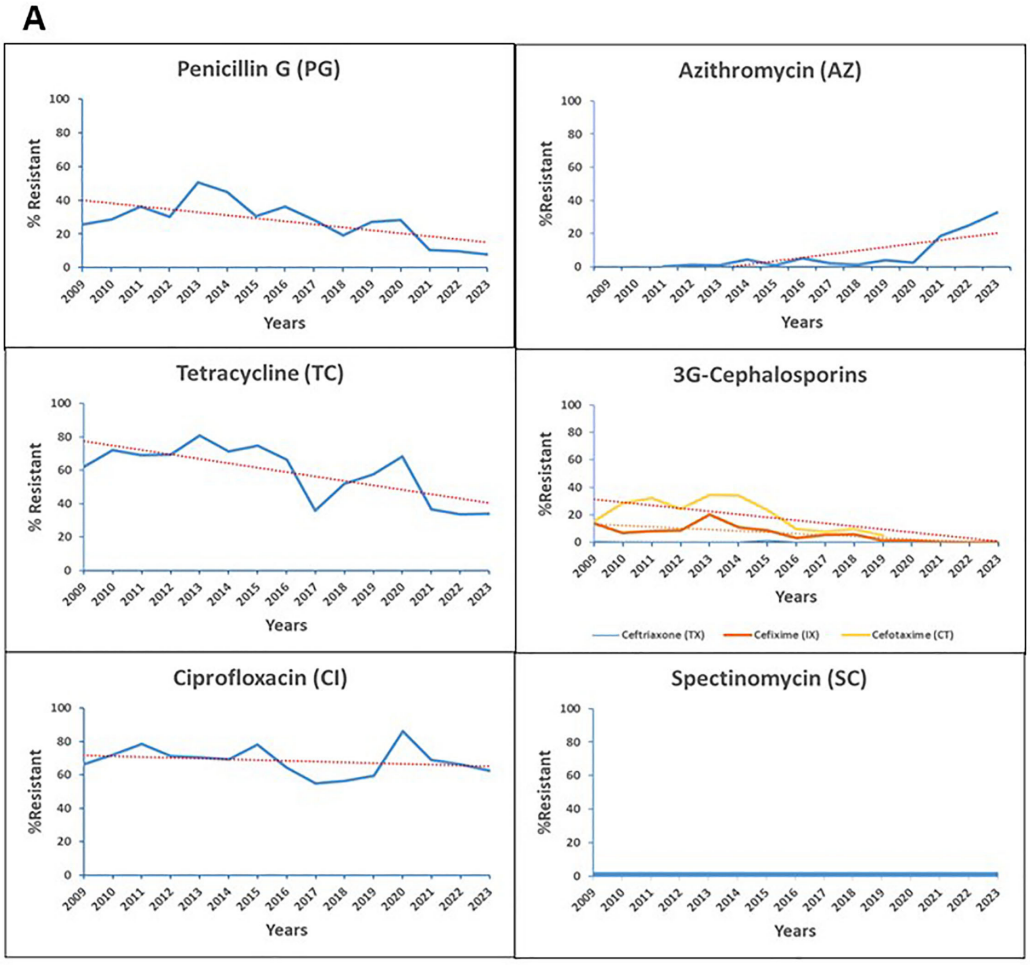
DISCUSSION

The epidemiological characteristics of the gonococcal population and the prevalence of resistant gonococci in Greece were studied.

Gonorrhoea cases were predominantly from males, with only a small number from females. A likely explanation is the asymptomatic nature of gonococcal infection in women, combined with limited proactive testing, leading to their under-representation in our sample.

The observed increasing trend in MSM patients within the studied population can be linked to various factors, including a rise in high-risk sexual behaviours—such as unprotected sex and multiple sexual partners—which have facilitated the transmission of gonorrhoea in this group.

A notable finding of this 15-year prospective survey is the gradual decline in the proportion of gonococci susceptible to all relevant antibiotics. Specifically, the percentage of isolates exhibiting full or intermediate susceptibility decreased from 64% during 2001–2004 to 41% in 2005–2008, and further declined to 18.3% in the current period (2009–2023).[5 6] This



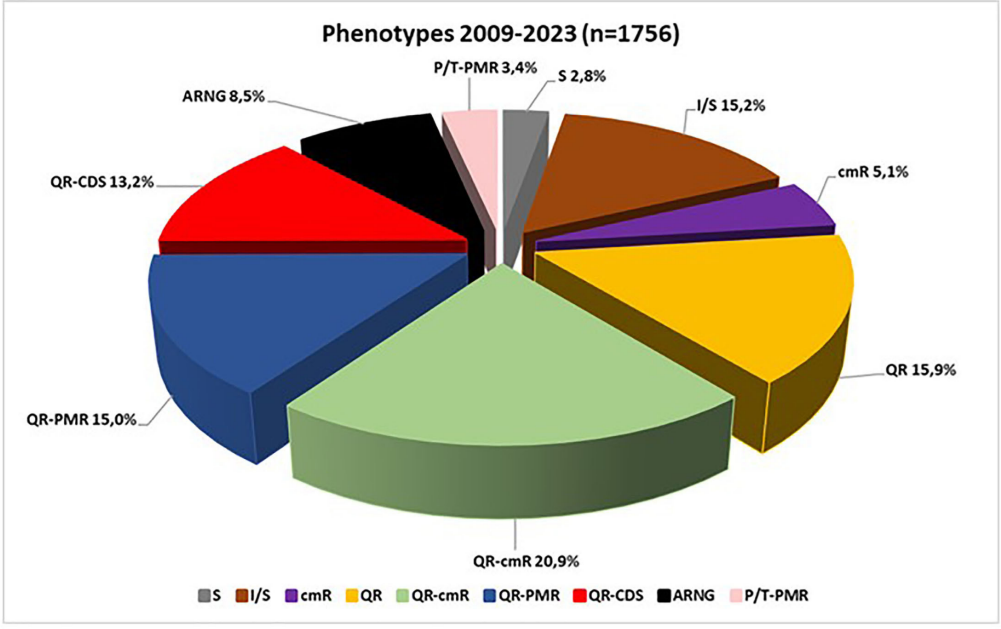


Figure 1 (A) Resistance rates and (B) resistance phenotypes of gonococcal isolates for the period 2009–2023. ARNG, resistance to azithromycin; cmR, chromosomal resistance to at least one of the antibiotics (penicillin, tetracycline, azithromycin); I/S, exhibited intermediate susceptibility (according to EUCAST) to at least one antibiotic; P/T-PMR, plasmid resistance to penicillin and/or tetracycline; QR, resistance to newer quinolones; QR-CDS, resistance to newer quinolones and at least one ESC; QR-cmR, resistance to newer quinolones combined with chromosomal resistance; QR-PMR, resistance to newer quinolones and plasmid-mediated resistance to penicillin and/or tetracycline; S, susceptibility to all antibiotics tested.

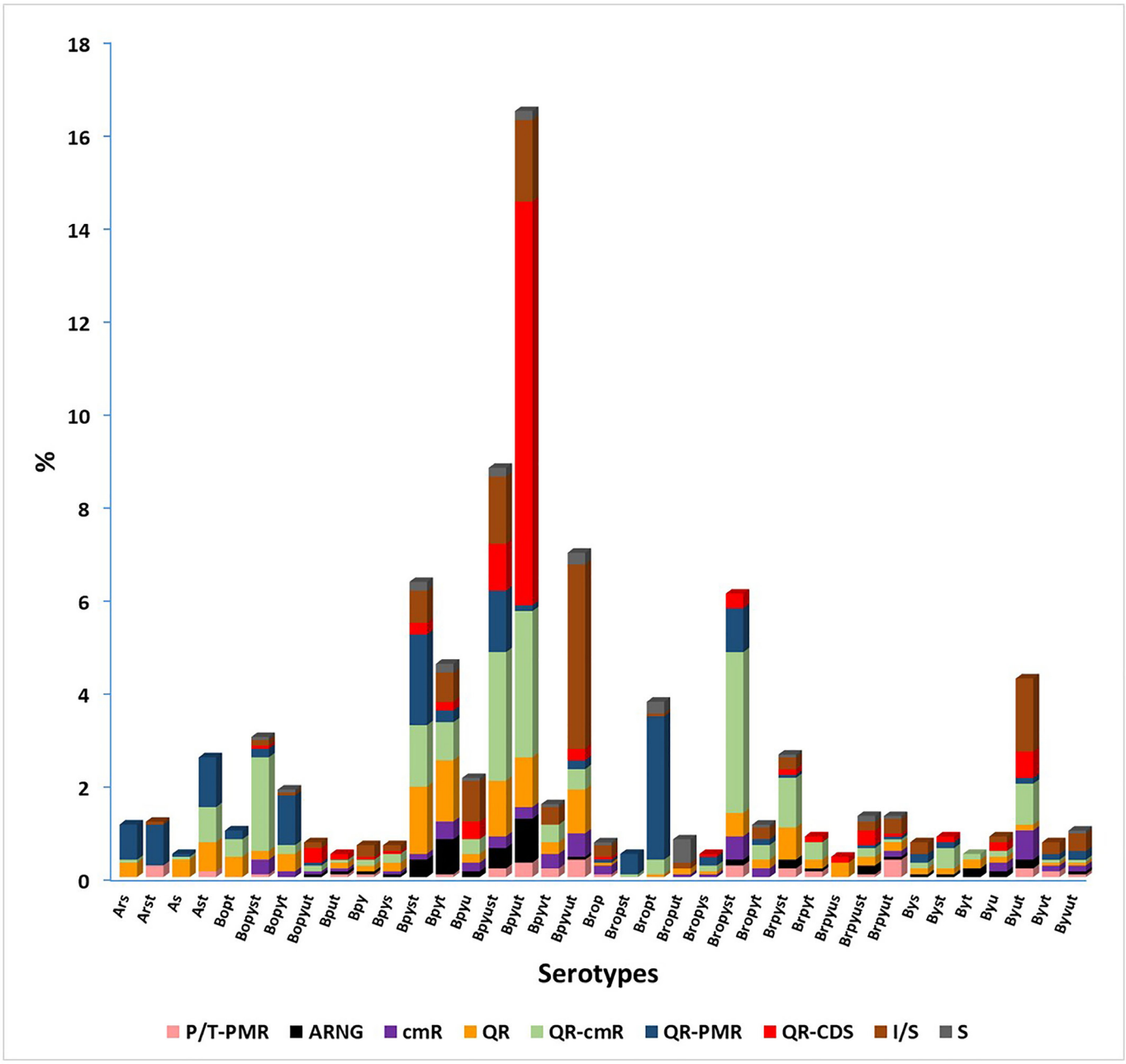
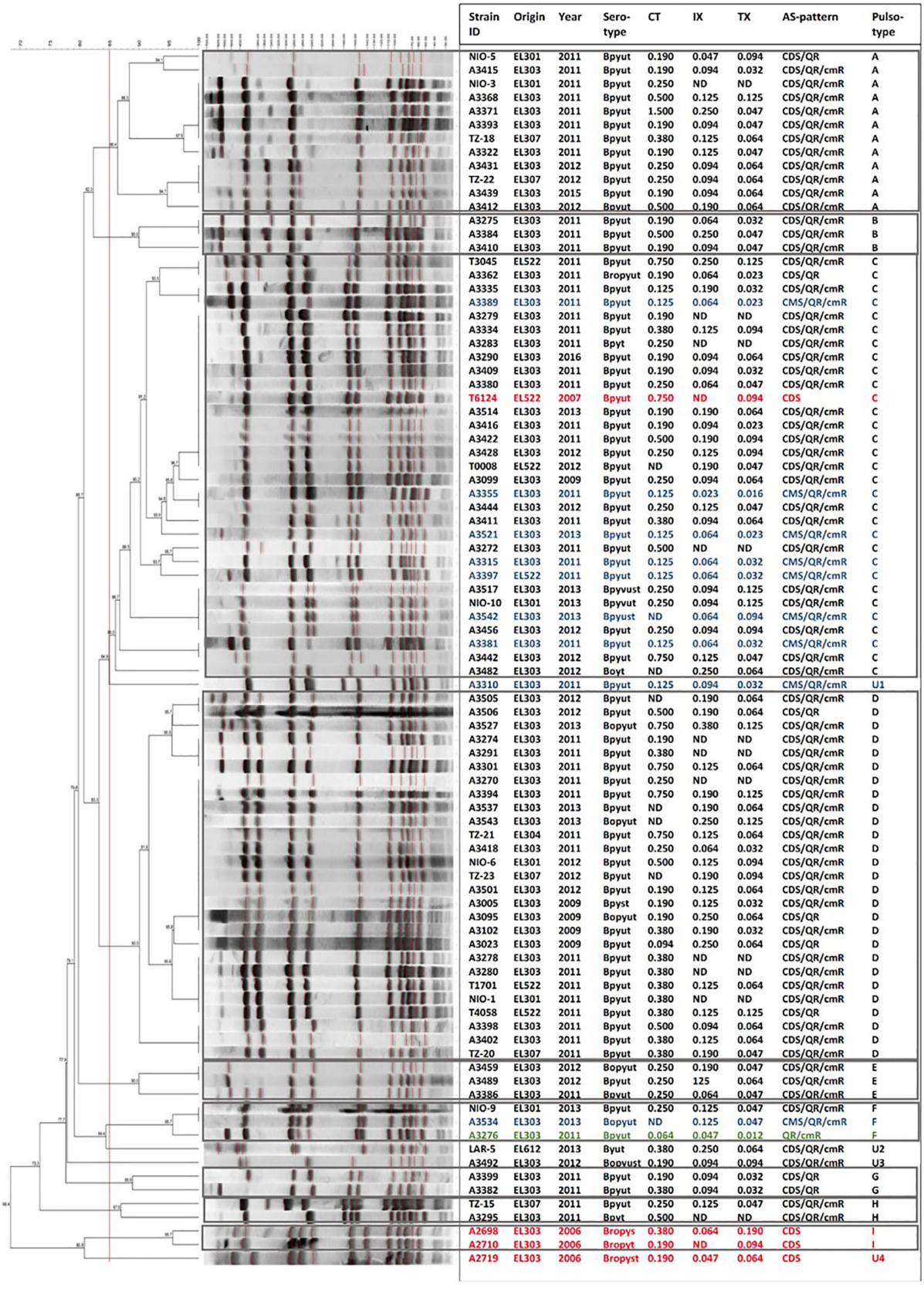


Figure 2 Distribution of *Neisseria gonorrhoeae* isolates in the most prevalent serotypes and resistance phenotypes, 2009–2023. ARNG, resistance to Azithromycin; cmR, chromosomal resistance to at least one of the antibiotics (penicillin, tetracycline, azithromycin); I/S, exhibited intermediate susceptibility (according to EUCAST) to at least one antibiotic; P/T-PMR, plasmid resistance to penicillin and/or tetracycline; QR, resistance to newer quinolones; QR-CDS, resistance to newer quinolones and at least one ESC; QR-cmR: resistance to newer quinolones combined with chromosomal resistance; QR-PMR, resistance to newer quinolones and plasmid-mediated resistance to penicillin and/or tetracycline; S, susceptibility to all antibiotics tested.

trend, substantiated by comprehensive epidemiological surveillance data, indicates a dramatic reduction in the sensitive gonococcal population over the last 20 years in Greece.

During the period 2009–2023, 22.4% of gonococci isolates were recorded with single drug resistance. Although fluctuations were observed during the study period, a decline in isolates with solely plasmid-mediated resistance to penicillin and/or tetracycline was seen. Specifically, the prevalence of isolates with only plasmid-mediated resistance to penicillin or tetracycline was 1.8% and 1.4%, respectively, lower than those recorded during the previous 8-year period 2001–2008.[5 6] On the contrary, the prevalence of isolates with sole resistance to fluoroquinolones (16%) and macrolides (3.2%) was increased, whereas in the previous period, those phenotypes accounted only for 1.2% and 0.2%, respectively of the total population. Additionally, isolates exhibiting only chromosomally mediated low levels of resistance to different groups of antibiotics showed a downward trend.

Multiresistant phenotypes accounted for 77.6% of gonococcus. The isolation of strains resistant to quinolones remains at consistently high levels (on average 68.8%) through the entire study period, with the majority of them being multiresistant. In contrast to previous periods, during 2009–2023, we witnessed an increase in quinolone-resistant strains with plasmid-mediated co-resistance (22.0%), a phenotype that was seen in 11.1% of



Strain ID	Origin	Year	Sero-type	CT	IX	TX	AS-pattern	Pulso-type
NIO-5	EL301	2011	Bpyut	0.190	0.047	0.094	CDS/QR	A
A3415	EL303	2011	Bpyut	0.190	0.094	0.032	CDS/QR/cmR	A
NIO-3	EL301	2011	Bpyut	0.250	ND	ND	CDS/QR/cmR	A
A3368	EL303	2011	Bpyut	0.500	0.125	0.125	CDS/QR/cmR	A
A3371	EL303	2011	Bpyut	1.500	0.250	0.047	CDS/QR/cmR	A
A3393	EL303	2011	Bpyut	0.190	0.094	0.047	CDS/QR/cmR	A
TZ-18	EL307	2011	Bpyut	0.380	0.125	0.064	CDS/QR/cmR	A
A3322	EL303	2011	Bpyut	0.190	0.125	0.047	CDS/QR/cmR	A
A3431	EL303	2012	Bpyut	0.250	0.094	0.064	CDS/QR/cmR	A
TZ-22	EL307	2012	Bpyut	0.250	0.094	0.064	CDS/QR/cmR	A
A3439	EL303	2015	Bpyut	0.190	0.094	0.064	CDS/QR/cmR	A
A3412	EL303	2012	Bpyut	0.500	0.190	0.064	CDS/QR/cmR	A
A3275	EL303	2011	Bpyut	0.190	0.064	0.032	CDS/QR/cmR	B
A3384	EL303	2011	Bpyut	0.500	0.250	0.047	CDS/QR/cmR	B
A3410	EL303	2011	Bpyut	0.190	0.094	0.047	CDS/QR/cmR	B
T3045	EL522	2011	Bpyut	0.750	0.250	0.125	CDS/QR/cmR	C
A3362	EL303	2011	Bropyut	0.190	0.064	0.023	CDS/QR	C
A3335	EL303	2011	Bpyut	0.125	0.190	0.032	CDS/QR/cmR	C
A3389	EL303	2011	Bpyut	0.125	0.064	0.023	CMS/QR/cmR	C
A3279	EL303	2011	Bpyut	0.190	ND	ND	CDS/QR/cmR	C
A3334	EL303	2011	Bpyut	0.380	0.125	0.094	CDS/QR/cmR	C
A3283	EL303	2011	Bpyt	0.250	ND	ND	CDS/QR/cmR	C
A3290	EL303	2016	Bpyut	0.190	0.094	0.064	CDS/QR/cmR	C
A3409	EL303	2011	Bpyut	0.190	0.094	0.032	CDS/QR/cmR	C
A3380	EL303	2011	Bpyut	0.250	0.064	0.047	CDS/QR/cmR	C
T6124	EL522	2007	Bpyut	0.750	ND	0.094	CDS	C
A3514	EL303	2013	Bpyut	0.190	0.190	0.064	CDS/QR/cmR	C
A3416	EL303	2011	Bpyut	0.190	0.094	0.023	CDS/QR/cmR	C
A3422	EL303	2011	Bpyut	0.500	0.190	0.094	CDS/QR/cmR	C
A3428	EL303	2012	Bpyut	0.250	0.125	0.094	CDS/QR/cmR	C
T0008	EL522	2012	Bpyut	ND	0.190	0.047	CDS/QR/cmR	C
A3099	EL303	2009	Bpyut	0.250	0.094	0.064	CDS/QR/cmR	C
A3355	EL303	2011	Bpyut	0.125	0.023	0.016	CMS/QR/cmR	C
A3444	EL303	2012	Bpyut	0.250	0.125	0.047	CDS/QR/cmR	C
A3411	EL303	2011	Bpyut	0.380	0.094	0.064	CDS/QR/cmR	C
A3521	EL303	2013	Bpyut	0.125	0.064	0.023	CMS/QR/cmR	C
A3272	EL303	2011	Bpyut	0.500	ND	ND	CDS/QR/cmR	C
A3315	EL303	2011	Bpyut	0.125	0.064	0.032	CMS/QR/cmR	C
A3397	EL522	2011	Bpyut	0.125	0.064	0.032	CMS/QR/cmR	C
A3517	EL303	2013	Bpyvust	0.250	0.094	0.125	CDS/QR/cmR	C
NIO-10	EL301	2013	Bpyvut	0.250	0.094	0.125	CDS/QR/cmR	C
A3542	EL303	2013	Bpyust	ND	0.064	0.094	CMS/QR/cmR	C
A3456	EL303	2012	Bpyut	0.250	0.094	0.094	CDS/QR/cmR	C
A3381	EL303	2011	Bpyut	0.125	0.064	0.032	CMS/QR/cmR	C
A3442	EL303	2012	Bpyut	0.750	0.125	0.047	CDS/QR/cmR	C
A3482	EL303	2012	Boyt	ND	0.250	0.064	CDS/QR/cmR	C
A3310	EL303	2011	Bpyut	0.125	0.094	0.032	CMS/QR/cmR	U1
A3505	EL303	2012	Bpyut	ND	0.190	0.064	CDS/QR/cmR	D
A3506	EL303	2012	Bpyut	0.500	0.190	0.064	CDS/QR	D
A3527	EL303	2013	Bopyut	0.750	0.380	0.125	CDS/QR/cmR	D
A3274	EL303	2011	Bpyut	0.190	ND	ND	CDS/QR/cmR	D
A3291	EL303	2011	Bpyut	0.380	ND	ND	CDS/QR/cmR	D
A3301	EL303	2011	Bpyut	0.750	0.125	0.064	CDS/QR/cmR	D
A3270	EL303	2011	Bpyut	0.250	ND	ND	CDS/QR/cmR	D
A3394	EL303	2011	Bpyut	0.750	0.190	0.125	CDS/QR/cmR	D
A3537	EL303	2013	Bpyut	ND	0.190	0.064	CDS/QR/cmR	D
A3543	EL303	2013	Bopyut	ND	0.250	0.125	CDS/QR/cmR	D
TZ-21	EL304	2011	Bpyut	0.750	0.125	0.064	CDS/QR/cmR	D
A3418	EL303	2011	Bpyut	0.250	0.064	0.032	CDS/QR/cmR	D
NIO-6	EL301	2012	Bpyut	0.500	0.125	0.094	CDS/QR/cmR	D
TZ-23	EL307	2012	Bpyut	ND	0.190	0.094	CDS/QR/cmR	D
A3501	EL303	2012	Bpyut	0.190	0.125	0.064	CDS/QR/cmR	D
A3005	EL303	2009	Bpyst	0.190	0.125	0.032	CDS/QR/cmR	D
A3095	EL303	2009	Bopyut	0.190	0.250	0.064	CDS/QR	D
A3102	EL303	2009	Bpyut	0.380	0.190	0.032	CDS/QR/cmR	D
A3023	EL303	2009	Bpyut	0.094	0.250	0.064	CDS/QR	D
A3278	EL303	2011	Bpyut	0.380	ND	ND	CDS/QR/cmR	D
A3280	EL303	2011	Bpyut	0.380	ND	ND	CDS/QR/cmR	D
T1701	EL522	2011	Bpyut	0.380	0.125	0.064	CDS/QR/cmR	D
NIO-1	EL301	2011	Bpyut	0.380	ND	ND	CDS/QR/cmR	D
T4058	EL522	2011	Bpyut	0.380	0.125	0.125	CDS/QR	D
A3398	EL303	2011	Bpyut	0.500	0.094	0.064	CDS/QR/cmR	D
A3402	EL303	2011	Bpyut	0.380	0.125	0.064	CDS/QR/cmR	D
TZ-20	EL307	2011	Bpyut	0.380	0.190	0.047	CDS/QR/cmR	D
A3459	EL303	2012	Bopyut	0.250	0.190	0.047	CDS/QR/cmR	E
A3489	EL303	2012	Bpyut	0.250	125	0.064	CDS/QR/cmR	E
A3386	EL303	2011	Bpyut	0.250	0.064	0.047	CDS/QR/cmR	E
NIO-9	EL301	2013	Bpyut	0.250	0.125	0.047	CDS/QR/cmR	F
A3534	EL303	2013	Bopyut	ND	0.125	0.047	CMS/QR/cmR	F
A3276	EL303	2011	Bpyut	0.064	0.047	0.012	QR/cmR	F
LAR-5	EL612	2013	Byut	0.380	0.250	0.064	CDS/QR/cmR	U2
A3492	EL303	2012	Bopyust	0.190	0.094	0.094	CDS/QR/cmR	U3
A3399	EL303	2011	Bpyut	0.190	0.094	0.032	CDS/QR	G
A3382	EL303	2011	Bpyut	0.380	0.094	0.032	CDS/QR	G
TZ-15	EL307	2011	Bpyut	0.250	0.125	0.047	CDS/QR/cmR	H
A3295	EL303	2011	Boyt	0.500	ND	ND	CDS/QR/cmR	H
A2698	EL303	2006	Bropys	0.380	0.064	0.190	CDS	I
A2710	EL303	2006	Bropyt	0.190	ND	0.094	CDS	I
A2719	EL303	2006	Bropyst	0.190	0.047	0.064	CDS	U4

Figure 3 Pulsed-field gel electrophoresis patterns obtained from analysis of *Neisseria gonorrhoeae* isolates with the restriction enzyme BcuI. The origin of the isolates is determined according to the geocode standards of the European Union (Nomenclature of Territorial Units for Statistics). Cefotaxime (CT), cefixime (IX) and ceftriaxone (TX) correspond to the minimum inhibitory concetration values (mg/L) determined by E-test. AS pattern corresponds to the resistance phenotypes. Black: cephalosporin decreased susceptibility (CDS) isolates from the period 2009 to 2019, blue: CMS isolates from the period 2009 to 2019, red: CDS isolates from the previous period 2005 to 2008, green: Bpyut isolate from the period 2009 to 2023 (control).

the QRNG isolates in earlier studies in Greece.[6] Moreover, a large proportion of quinolone-resistant isolates also exhibited decreased susceptibility to ESCs and chromosomally mediated low-level resistance.

Azithromycin resistance was recorded at low rates; however, an increasing trend was observed until 2020. After 2021, a shift has been seen to MICs above the ECOFF (>1 mg/L) leading to higher resistance rates and the appearance of azithromycin-resistant isolates with multiresistant phenotypes, exhibiting co-resistance to quinolones. The emergence of isolates with high-level resistance to azithromycin was recorded in 2023. This change has been reported from several European countries.[19] It can be hypothesised that the introduction of azithromycin as the second antibiotic in dual therapeutic regimens for *N. gonorrhoeae* has facilitated the emergence of resistant clones in the gonococcal population.[20] The latter jeopardises the use of azithromycin as antimicrobial agents for the treatment of *N. gonorrhoeae*, thus, the national guidelines have been updated in 2023 by the National Public Health Organisation in Greece. Specifically, azithromycin is not recommended as a first-line treatment for gonorrhoea and should only be used after susceptibility testing confirms its effectiveness.

During the first years of the studied period, the proportion of CDS isolates was relatively stable. However, it is worth mentioning that a decline in the incidence of ceftriaxone-resistant *N. gonorrhoeae* isolates has been observed since 2016, probably due to the regression of circulating CDS clones that were predominant in Greece in the previous period. The main CDS clones circulating in Greece during the period 2009–2019 were of serotype Bpyut/pulsotypes A, B, C and D, for which a decline was observed after 2016. After 2016 and until 2020, the isolates of the Bpyut serovar exhibited susceptible/intermediate susceptible or quinolone-resistant phenotypes, and CDS-Bpyut were only sporadically isolated. Since 2021, we have witnessed the disappearance of CDS isolates. The aforementioned resistance/clonal characteristics indicate that, during the studied period, resistance to ESCs has continuously decreased, most probably due to the spread of other gonococcal clones, as clearly indicated by serotyping and PFGE data.

A limitation of the present study is the inherent challenge in drawing comparisons with existing studies, given that only epidemiological and microbiological data were available throughout the 15-year study period. Due to the inconsistent availability of genomic data during these years, such data were excluded from the analysis.

The shaping of the gonococcal population in Greece could be the result of a combination of events, the continuous replacement of the predominating clones and introduction of new ones in the gonococcal population with different resistance characteristics. In summary, the gonococcal population showed a continuous change in the predominating bacterial clones as well as in resistance phenotypes during the study period in comparison with the previous ones.[5 6] This is likely due to alterations of the clonal lineages that are prevailing. The changes in gonococcal resistance to antibiotics as found here underline the need for systematic monitoring of the antibiotic susceptibility trends of this pathogen. Apart from the epidemiological monitoring of *N. gonorrhoeae* cases regarding the dissemination of specific bacterial clones in the community, surveillance data for antimicrobial susceptibility are essential in order to guide empirical therapy of gonorrhoea both at national and international levels.

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Contributors VM (guarantor): conceptualisation, ES, ET and VM; isolate collection, A-DP, S-EC and IMo; experimental work, ES, A-DP, ET and VM; data analysis, ES, MP, IMa, AB, ET and VM; writing—original draft preparation, ES; writing—review and editing, AB, ET and VM; visualisation; ES and VM; funding acquisition, IMa, ET and VM. All authors contributed to the writing of the manuscript and approved the final submitted version.

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Competing interests None declared.

Patient consent for publication Written consent was not required, as data were collected in accordance with Art. 6 par. 1e (Processing is necessary for the performance of a task carried out in the public interest) and Art. 9 par. 2j (Processing is necessary for scientific research purposes) of the GDPR, within the context of epidemiological surveillance conducted by the National Reference Center for *Neisseria gonorrhoeae*.

Ethics approval This study was approved by the Committee of Research Ethics of University of West Attica (ref. no. 37935-11/05/2021) and the Committee of Bioethics of the Hellenic Pasteur Institute (ref. no. 4320/10-07-2024). Data were managed in accordance with the national and European Union laws. The Hellenic Pasteur Institute complies with the requirements for the protection of individuals with regard to the processing of personal data (GDPR EU/2016/679) and the Greek Legislation (4624/2019).

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Data availability statement Data are available on reasonable request. Sequencing data are available in GenBank. Data are available on reasonable request.

ORCID iDs
Apostolos Beloukas http://orcid.org/0000-0001-5639-0528
Vivi Miriagou http://orcid.org/0000-0003-3905-0315

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