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Carballo R, Povoa MC, Abad R, et al. Large Increase in Azithromycin-Resistant Neisseria gonorrhoeae in Northern Spain. Microbial Drug Resistance. 2022;28(1):81-86. doi:10.1089/mdr.2020.0594

which has been published in final form at:
https://doi.org/10.1089/mdr.2020.0

Large increase in azithromycin-resistant *Neisseria gonorrhoeae* in Northern Spain

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ABSTRACT

The aim of this study was to characterize the evolution of gonorrhoea in the general population by correlating epidemiological, genotypic and antimicrobial resistance data of *Neisseria gonorrhoeae* isolates collected in northern Spain from 2014 to 2018. One hundred ninety-four strains underwent antimicrobial susceptibility testing and were genetically analyzed by *N. gonorrhoeae* multiantigen sequence typing.

Increasing cases of gonococcal infections have been observed after 2015. Most occurred in male with urethritis. Sequence type (ST)-9972 and ST-1576, the predominant genotypes identified, have not been previously described as epidemic clones. Of great concern was the significant increase in azithromycin-resistant *N gonorrhoeae*. More than 30% of these isolates were obtained from men who have sex with men (MSM). ST-12302 was the most prevalent clone among the azithromycin-resistant strains, and was also resistant to penicillin, ciprofloxacin, and tetracycline. This multidrug-resistant clone was exclusively isolated from MSM during 2018. The incidence rates of gonorrhoeae and azithromycin-resistant *N. gonorrhoeae* have significantly increased due to emergence of new clones. ST-12302 has recently been recognized as an epidemic clone; therefore, its surveillance could be the key in controlling further dissemination of azithromycin resistance. These data highlight the need to perform local studies to update treatment guidelines and reinforce preventive measures against gonorrhea.

INTRODUCTION

Neisseria gonorrhoeae is one of the most frequent causes of sexually transmitted infections. *N. gonorrhoeae* has acquired resistance to all recommended antibiotics and is considered an urgent global threat by the World Health Organisation (WHO).[1] Some of the current European guidelines, including the Spanish, recommend a dual therapy with ceftriaxone (0.5-1g, intramuscular injection) plus azithromycin (1-2 g, orally) as the first-line treatment for uncomplicated gonorrhea.[2,3] A single-dose regimen using this combination prevent abandonment of treatment and emergence of resistance. Moreover, dual therapy is also effective against *Chlamydia trachomatis.* Despite these measures, outbreaks of *N. gonorrhoeae* resistant to azithromycin and combined resistance to ceftriaxone and azithromycin have been described, suggesting that this dual-antibiotic regimen may not be effective in the long term.[4,5] Due to the increase in azithromycin resistance, the Centers for Disease Control and Prevention (CDC) have recently recommended a single 500 mg intramuscular dose of ceftriaxone for uncomplicated gonorrhea and removal of azithromycin from the recommended regimen.[6] Therefore, epidemiological surveillance programs have been launched to gain a better understanding of the emergence and propagation of certain *N. gonorrhoeae* strains through their genotypic and phenotypic characterization.[1] This report examines traits of *N. gonorrhoeae* isolates from patients attending the Health Area of Vigo (northern Spain), providing relationships between their sequence-types (STs), antimicrobial resistance, and epidemiological data.

MATERIALS AND METHODS

Between 2014 and 2018, a total of 194 *N. gonorrhoeae* strains were isolated in the Clinical Microbiology department of the University Hospital Complex of Vigo (Spain), which consists of 2 hospitals and 24 community medical centers and serves 450,000 inhabitants. Only one isolate per patient was considered.

One hundred seventy-nine patients were males and fifteen females. The median The median age

was 34 years (16–71) for males and 30 years (16–69) for females. Thirty-nine cases were found in 2014 (8.1 cases/100,000 inhabitants), 16 in 2015 (3.5/100,000), 25 in 2016 (5.3/100,000), 37 in 2017 (8.2/100,000), and 77 in 2018 (16.3/100,000).

A total of 147 patients were attended at community medical centers, 37 at the internal medicine department and 10 at the gynecology department. Those assisted at the Internal Medicine department were men who have sex with men (MSM). Information on sexual orientation was not available for the remaining patients.

The specimen types were 163 from the urethra, 14 from the cervix, 11 from the rectum, 5 from the pharynx, and 1 from joint fluid. Swabs were cultured on Thayer-Martin medium (PolyviteX VCAT3 agar, BioMérieux, France) and incubated for 24–48 hours at 35–37ºC in a 5% CO2-enriched atmosphere. NG colonies were identified using the cytochrome oxidase test, the NH API system (BioMérieux) biochemical tests (BioMérieux), and mass spectrometry (MALDI-TOF, Brucker, Germany). *N. gonorrhoeae* strains were subcultured to obtain fresh colonies for antimicrobial susceptibility testing and frozen at -80ºC in trypticase soy broth with 20% glycerol.

The minimum inhibitory concentrations (MICs) of penicillin, ciprofloxacin, tetracycline, ceftriaxone, and azithromycin were determined by Etest (BioMérieux) on GC agar with 1% IsoVitaleX enrichment (BD chocolate agar, Becton Dickinson, USA). MICs were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints,[7] using ATCC 49226 as the quality control strain.[1,8]

N. gonorrhoeae isolates (one per patient) were molecular characterized by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) in the Neisseria Reference Laboratory of the National Centre for Microbiology (ISCIII). NG-MAST was performed by sequencing two variable genes (*por* and *tbpB*) as described previously by Martin *et al*.[9]

Primer (Sigma Genosys) and PCR condition information are available on http://www.ng-mast.net/misc/info2.asp. PCR reactions were carried out using illustra PuReTaq Ready-To-Go (GE Healthcare) on an Applied Biosystems™ 2720 Thermal Cycler. The sequencing was carried out on

an ABI PRISM 3100 (Applied Biosystems) sequencer using BigDye v3.1 cycle sequencing (Applied Biosystems). The forward and reverse sequences of each strain were aligned, edited, and trimmed to a set length (the *por* sequence is trimmed to 490 bp, starting from the following conserved region spanning from pre-loop 3 to loop 6: TTGAA; and the *tbpB* sequence is trimmed to 390 bp, starting from the conserved region: CGTCTGAA). The sequences were compared with the NG-MAST databases to assign alleles and STs.

The Mantel-Haenszel chi-square test for trend was used to identify changes over time in antimicrobial susceptibility. Mean MICs were calculated as geometric means per year. Time trends were assessed by performing a linear regression analysis of the logarithmic MICs.

Fisher's exact test was used to determine differences between years. Significance was set at $p < 0.05$. Statistical analysis was performed with SPSS software v19.0.

RESULTS

The distribution of MIC values by year for azithromycin, penicillin, tetracycline, ciprofloxacin, and ceftriaxone is shown in Fig. 1 (panels a, b, c, d, and e, respectively). For azithromycin, the highest proportion of isolates had an MIC of 0.065–0.125 mg/L. Over time, the mean MIC for azithromycin increased significantly ($p < 0.001$) from 0.140 mg/L in 2014 to 0.380 mg/L in 2018 (Table 1). Resistance to azithromycin (epidemiological cutoff, ECOFF >1 mg/L) was observed in 7.2% (14/194) of the isolates, with MICs varying from 1.5 to 6 mg/L; a significant increase was found in the 5-year period ($p = 0.012$), with the highest proportion of isolates with resistance to azithromycin (15.6%) in 2018 (Fig. 2). Six of these 14 strains were obtained from extragenital samples of MSM, four from the rectum and two from the pharynx.

The trend analysis shows that there is no significant difference in mean MIC for penicillin and tetracycline between years (Table 1). Resistance (MIC >1mg/L) or reduced susceptibility to penicillin (MIC= 0.096–1mg/L) and resistance to tetracycline (MIC >1mg/L) were found in 79.9%(155/194) and 74.7% (145/194) of the isolates, respectively (Fig. 2).

For ciprofloxacin, the biggest proportion of isolates tested had an MIC ≤0.016 mg/L and an MIC ≥1.5 mg/L (Fig. 1). Despite the drop in ciprofloxacin resistance (MIC >0.06 mg/L) observed between 2014 (82.1%) and 2016 (28%) ($p < 0.001$), it appears to have increased in 2017 (35%) and 2018 (63.6%) (Fig. 2). Nevertheless, trend analysis showed that the mean MIC for ciprofloxacin decreased significantly ($p = 0.013$) from 1.767 mg/L in 2014 to 0.313 mg/L in 2018.

For ceftriaxone, the biggest proportion of isolates tested had an MIC ≤0.016 mg/L and the percentage of strains with higher MICs appears to have decreased over time (Fig. 1). Moreover, the mean MIC for ceftriaxone decreased significantly ($p = 0.014$) from 0.020 mg/L in 2014 to 0.017 mg/L in 2018 (Table 1). Although all ceftriaxone MIC values were under the resistance breakpoint (MIC >0.125 mg/L), two isolates with decreased susceptibility were identified, one in 2015 and the other in 2016 (MIC = 0.125 mg/L).

Molecular characterization was performed on the 194 strains, resulting in the identification of 77 STs. The two most frequent clones were isolated in different periods, ST-9972 was detected from 2014 to 2016 and ST-15769 from 2017 to 2018.

Clones with more than two isolates and their general characteristics are shown in a table of Supplementary Table S1. Notably, 14 isolates belonging to 6 different STs were resistant to all antimicrobials tested, except ceftriaxone. One of these multidrug-resistant clones, ST-12302, was the most prevalent clone among the azithromycin-resistant strains and was exclusively obtained from MSM in 2018. Characteristics of the multidrug-resistant clones and the two isolates with elevated ceftriaxone MICs are shown in Table 2.

DISCUSSION

An increasing prevalence of gonococcal infections was observed in the health care area of Vigo (northern Spain) during 2015–2018, with 3.5 cases per 100,000 inhabitants in 2015 and 14.6 cases per 100,000 inhabitants in 2018. Most of these infections occurred in male patients with urethritis (92.3%), similar to data reported by Ibargoyen *et al.*[10] and the European Centre for Disease

Prevention and Control (ECDC).[11] Nevertheless, the incidence of infections observed in 2018 was lower than that noted in other areas of northern Spain (45.6 cases/100,000 population) and that reported by the ECDC in 2018 (26.4 cases/100,000 population).[10,11]

Of greater concern was a significant increase in mean MIC for azithromycin ($p < 0.001$), and in the proportion of isolates with resistance to azithromycin ($p = 0.012$) in the 5-year period, reaching 15.6% in 2018, which exceeds previously reported rates from Spain and Europe.[8,10,11] Most azithromycin-resistant strains were also resistant to the other antimicrobials tested, except for ceftriaxone. Although these strains remain treatable, dual therapy treatment failure has been reported in several international settings because of the emergence of ceftriaxone-resistant and high-level azithromycin resistant *N. gonorrhoeae*.[4,5,12] Resistance to ceftriaxone has also been sporadically detected in Spain.[8,13] In response to this concern, the United Kingdom and the United States have recently eliminated dual therapy from its guidelines, recommending ceftriaxone monotherapy and reserving azithromycin as a second-line treatment for gonorrhea, to reduce selective pressure in the population.[6,12] A similar update in the treatment guidelines would likely benefit our health care area.

Of note, more than 30% of azithromycin-resistant gonococci were isolated from extragenital sites of MSM. Accordingly, it is well known that the pharynx and rectum are important reservoirs for *N. gonorrhoeae* transmission in this group of patients. Pharyngeal *N. gonorrhoeae* infection is likely to be critical in both the epidemiology of gonorrhea and the propagation of resistance in MSM, as well as in heterosexual individuals.[1,12] Thus, screening of all the exposed anatomical sites on those at increased risk (MSM and men with risky sexual practices) should be adopted in primary care, as a large fraction of these infections will be missed if only urogenital testing is performed.

The resistance rates of penicillin, ciprofloxacin, and tetracycline were greater than 30%, similar to the rest of Spain and Europe.[10,11,13] Interestingly, a tendency of decreasing rates in ciprofloxacin resistance was observed between 2014 and 2016 ($p < 0.001$), likely due to its replacement by the ceftriaxone-azithromycin treatment, which started in 2014. Unfortunately, data on medical

prescriptions were not available to confirm this hypothesis.

The identification of 77 different STs revealed considerable clonal diversity among the *N. gonorrhoeae* isolates from this area. Such heterogeneity was also found in other studies over the last 5 years.[10,14] To our knowledge, the most prevalent genotypes, ST-9972 and ST-15769, have not been previously described in Spain or Europe, which might suggest that they are endemic clones to this region. In contrast, the internationally spread clones ST-1407 and ST-2992 were represented by four strains only, illustrating the instability of the gonococcal population in this area and why constant monitoring of gonorrhea is essential.[8,10,14]

In our study, azithromycin resistance was detected in nonclosely related strains, evidencing a polyclonal origin of emergence of resistance. Nevertheless, ST-12302 predominance indicates a possible spread among MSM in 2018. Moreover, the multidrug-resistant ST-12302 has been newly recognized as an epidemic clone in Canada[15]; thus, it could become a public health threat if antibiotic selection pressure remains, similar to other cases of transmission of existing azithromycin-resistant clones.[16]

These findings support the importance of performing local studies for tracking the antimicrobial resistance and spread of different clonal lines of *N. gonorrhoeae* and are useful to update treatment guidelines against gonorrhea and to reinforce preventive actions aimed at changing high-risk behaviors in particular risk groups.

Authors' Contributions

Designed the study: R.C., M.C.P., M.A., and C.P. Collected and processed the isolates: R.C., M.C.P., M.A., A.S., R.A., C.N., and E.M. Wrote the article: R.C., M.C.P., M.A., and C.P. Revision of article: all authors. Guarantors: R.C. and C.P.

Disclosure Statement

No competing financial interests exist.

Funding Information

This work was supported by the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC), call for research projects in Infectious Diseases and Clinical Microbiology 2018 (to M.C.P.).

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Table 1. Geometric Mean Minimum Inhibitory Concentration per year for Azithromycin, Penicillin, Ciprofloxacin, Tetracycline, and Ceftriaxone of *Neisseria gonorrhoeae* isolates, 2014–2018.

Year	No. of isolates	Geometric mean MIC (mg/L) (95% CI)				
		Penicillin	Tetracycline	Ciprofloxacin	Ceftriaxone	Azithromycin
2014	39	0.446 (0.257-0.772)	1.392 (0.982-1.974)	1.767 (0.621-5.021)	0.020 (0.017-0.023)	0.141 (0.101-0.196)
2015	16	0.401 (0.165-0.973)	1.499 (0.604-3.721)	1.133 (0.146-8.767)	0.023 (0.013-0.039)	0.101 (0.068-0.150)
2016	25	0.185 (0.115-0.300)	0.477 (0.234-0.975)	0.023 (0.006-0.101)	0.018 (0.015-0.022)	0.119 (0.094-0.150)
2017	37	0.124 (0.079-0.197)	0.511 (0.295-0.887)	0.031 (0.009-0.100)	0.018 (0.016-0.020)	0.087 (0.062-0.122)
2018	77	0.311 (0.202-0.480)	1.384 (0.897-2.136)	0.313 (0.131-0.749)	0.017 (0.016-0.018)	0.384 (0.289-0.509)

Total number of isolates = 194.

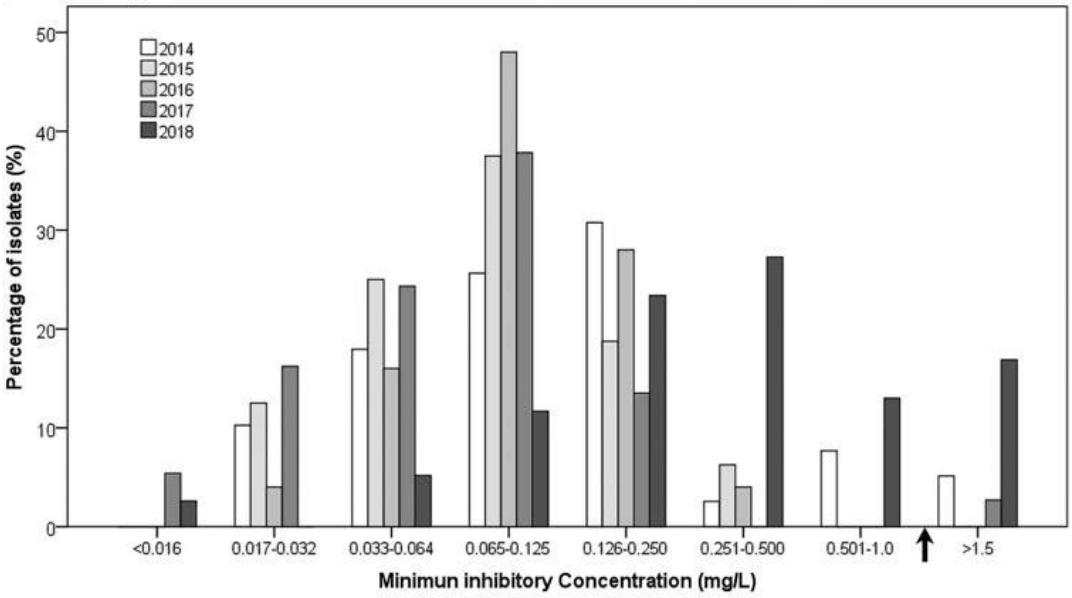
CI, confidence interval; MIC, minimum inhibitory concentration.

Table 2. Characteristics of Azithromycin-Resistant *Neisseria gonorrhoeae* and the two isolates with decreased Ceftriaxone susceptibility

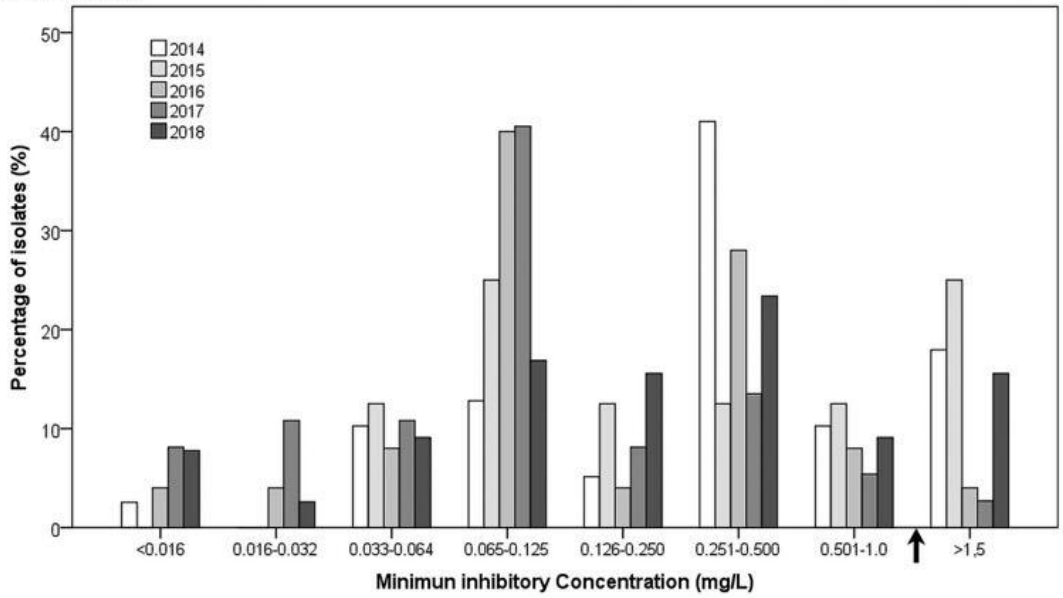
				MIC (mg/L)				
NG-MAST	*porB* allele	*tbpB* allele	Year	Penicillin[a]	Tetracycline[b]	Ciprofloxacin[c]	Ceftriaxone[d]	Azithromycin[e]
ST-11727	3031	39	2014	0.125	4	12	0.016	1.5
ST-6974[f]	**908**	**137**	**2015**	**3**	**2**	**1.5**	**0.125**	**0.064**
ST-9972[f]	**5879**	**110**	**2016**	**0.38**	**1.5**	**4**	**0.125**	**0.12**
ST-7638	536	894	2017	0.094	16	0.6	0.094	6
ST-5622	3411	110	2018	0.38	4	4	0.016	1.5
ST-6765	2078	29	2018	0.094	1.5	0.016	<0.016	2
ST-6765	2078	29	2018	0.5	1.5	0.004	<0.016	2
ST-12302	908	267	2018	0.38	2	32	<0.016	1.5
ST-12302	908	267	2018	0.19	2	4	<0.016	2
ST-12302	908	267	2018	0.38	2	6	<0.016	4
ST-12302	908	267	2018	0.75	2	12	<0.016	3
ST-12302	908	267	2018	0.094	1.5	>32	<0.016	6
ST-17371	5314	29	2018	0.19	1	0.016	<0.016	6
ST-18004	6720	121	2018	0.38	0.75	0.002	<0.016	4
ST-18370	7738	29	2018	0.125	1	0.008	<0.016	6
ST-18370	7738	29	2018	0.094	1	0.006	<0.016	4

[a]Resistance defined as MIC >1 mg/L and reduced susceptibility as MIC = 0.094–1 mg/L.
[b]Resistance defined as MIC >1 mg/L.
[c]Resistance defined as MIC >0.06 mg/L.
[d]Resistance defined as MIC >0.125 mg/L.
[e]Resistance defined as epidemiological cutoff, ECOFF >1 mg/L (EUCAST 2021; version 11.0).
[f]The two isolates with decreased ceftriaxone susceptibility are highlighted in bold.
MIC, minimum inhibitory concentration; NG-MAST, Neisseria gonorrhoeae multiantigen sequence typing.

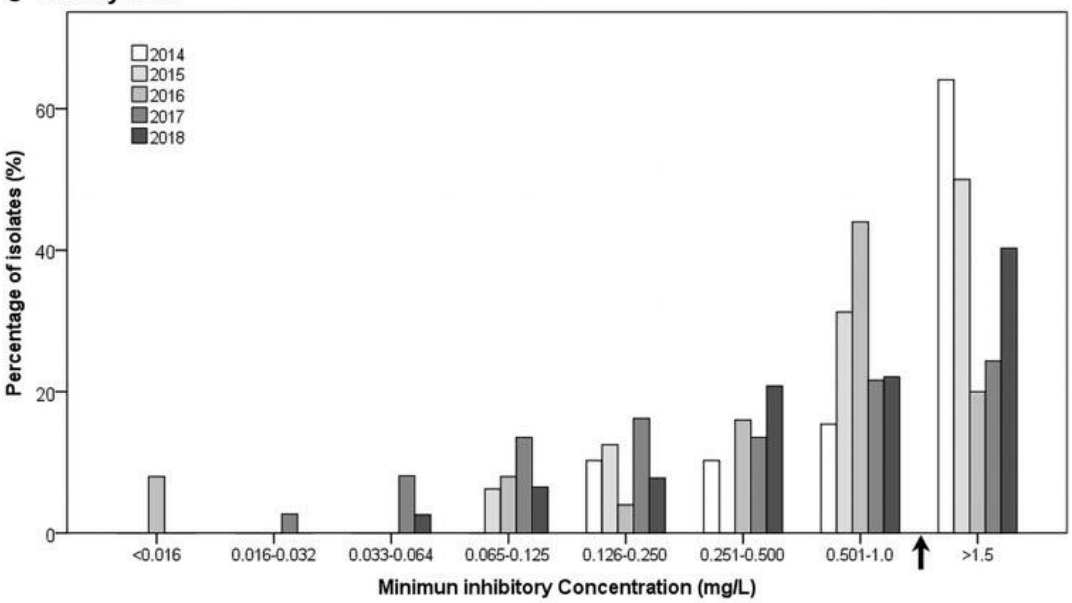
a Azithromycin



b Penicillin



c Tetracycline



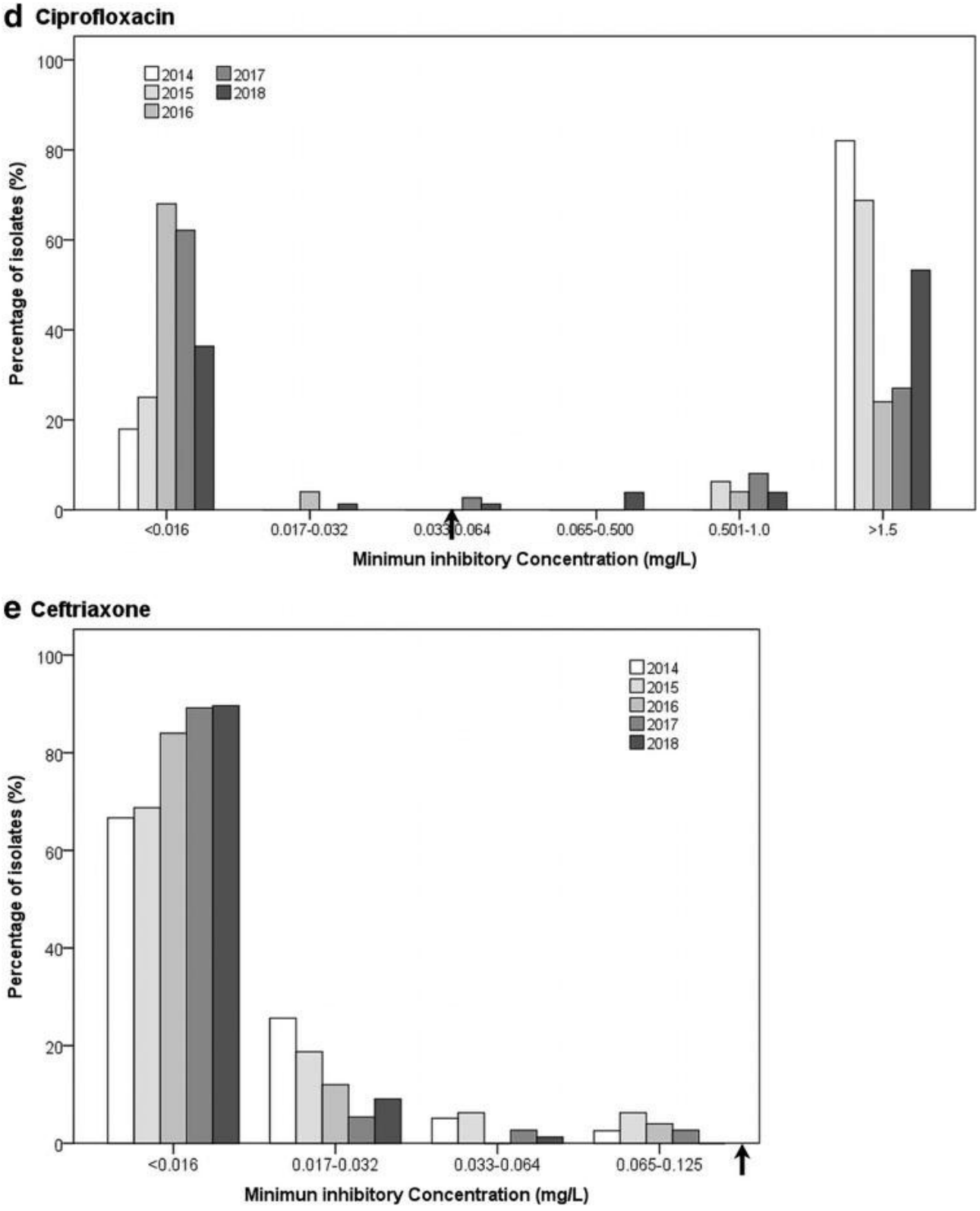


Figure 1. Distribution of MIC values for (a) azithromycin, (b) penicillin, (c) tetracycline, (d) ciprofloxacin, and (e) ceftriaxone of *Neisseria gonorrhoeae* by year, 2014–2018 (n = 194 isolates). The arrows indicate the threshold for antimicrobial resistance according to the European Committee on Antimicrobial Susceptibility Testing guideline (2021).

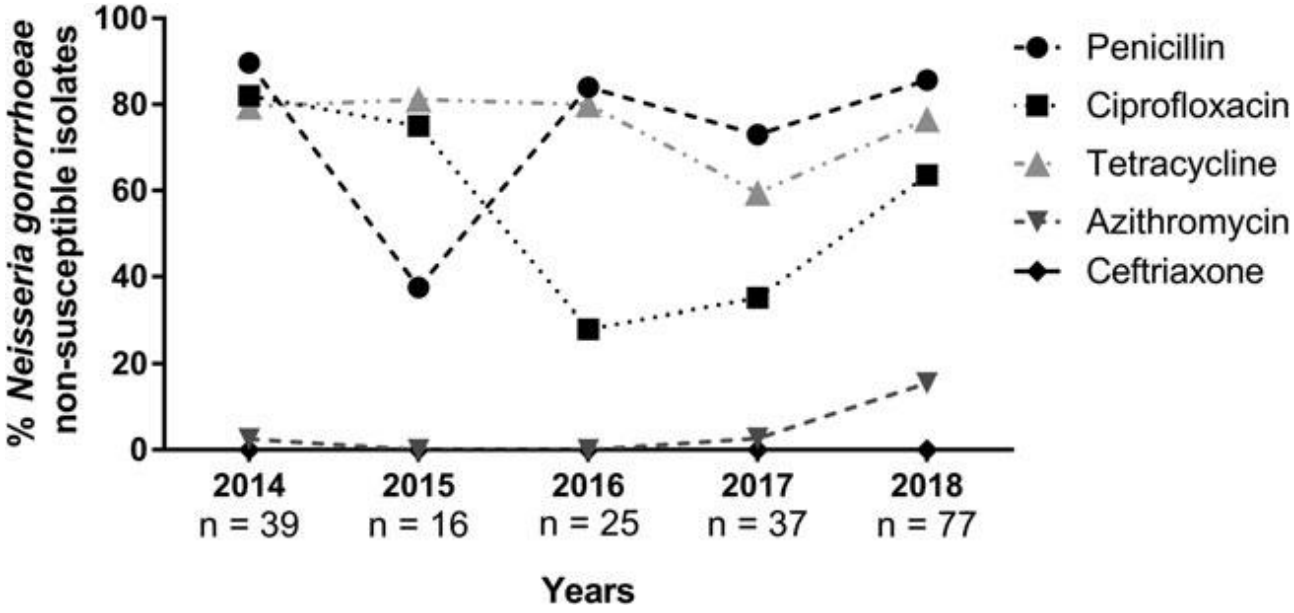


Figure 2. Evolution of nonsusceptible *Neisseria gonorrhoeae* isolates according to the year of isolation (n = 194). Nonsusceptible isolates are those with MICs >0.06 and ≤1 mg/L for penicillin, resistant to penicillin (MIC >1 mg/L), resistant to azithromycin (ECOFF >1 mg/L), resistant to tetracycline (MICs >1 mg/L), resistant to ciprofloxacin (MICs >0.06 mg/L), and resistant to ceftriaxone (MICs >0.125 mg/L).