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Antibiotic susceptibility and genotypic characterization of *Neisseria gonorrhoeae* isolates in the Comunidad Valenciana (Spain): GONOVig project



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ABSTRACT

Introduction: The increase in sexually transmitted infections (STI) caused by *Neisseria gonorrhoeae* (NG) worldwide, together with the decrease in antibiotic susceptibility, forced us to understand the epidemiology of gonococcal infection.

Methods: The GONOVig project analyzed, comparatively following CLSI and EUCAST criteria, the antibiotic susceptibility of 227 NG strains collected in thirteen representative hospitals of the Valencia Community (CV) between 2013 and 2018. Additionally, molecular typing of 175 strains using the NG multi-antigen sequence typing technique (NG-MAST) was performed.

Results: High rates of resistance to tetracycline (38.2% by CLSI and 50.9% by EUCAST) and ciprofloxacin (49.1% CLSI and 54% EUCAST), and low percentages of resistance to spectinomycin (0%), cefixime (0.5% CLSI but 5.9% EUCAST), and ceftriaxone (1.5% CLSI and 2.4% EUCAST) were detected. Azithromycin resistance was 6% (both CLSI and EUCAST). Molecular analysis revealed the presence of 86 different sequence types (ST), highlighting ST2992 (7.4%), ST3378 (6.9%), ST2400 (4.6%) and ST13288 (6.9%), which was associated with resistance to cefixime ($P = .031$). The main genogroups (G) were G1407 (13.1%), G2992 (10.3%), G2400 (6.3%) and G387 (3.4%). G1407 and G2400 were associated with resistance to ciprofloxacin ($P < .03$).

Conclusion: Low resistance to ceftriaxone, a worrying resistance to azithromycin and a wide variety of circulating sequence types have been detected, some of which show correlation with certain resistance profiles.

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Susceptibilidad antibiótica y caracterización genotípica de aislados de *Neisseria gonorrhoeae* circulantes en la Comunidad Valenciana (España): proyecto GONOVig

RESUMEN

Introducción: El aumento de las infecciones de transmisión sexual producidas por *Neisseria gonorrhoeae* (NG) a nivel mundial, junto con la disminución de la susceptibilidad antibiótica, obliga a profundizar en la epidemiología de la infección gonocócica.

Métodos: El proyecto GONOVig analizó, comparativamente siguiendo criterios CLSI y EUCAST, la sensibilidad antibiótica de 227 cepas de NG recogidas en trece hospitales representativos de la Comunidad Valenciana (CV) entre los años 2013 y 2018. Adicionalmente, se pudo realizar la tipificación molecular de 175 cepas mediante la técnica NG multi-antigen sequence typing (NG-MAST).

Palabras clave:

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Resultados: Se detectaron elevadas tasas de resistencia a tetraciclina (38,2% por CLSI y 50,9% por EUCAST) y ciprofloxacino (49,1% CLSI y 54% EUCAST), y bajos porcentajes de resistencia a espectinomicina (0%), cefixima (0,5% CLSI pero 5,9% EUCAST) y ceftriaxona (1,5% CLSI y 2,4% EUCAST). La resistencia a azitromicina fue del 6% (tanto CLSI como EUCAST). El análisis molecular reveló la presencia de 86 secuenciotipos (ST) distintos, destacando el ST2992 (7,4%), ST3378 (6,9%), ST2400 (4,6%) y ST13288 (6,9%) el cual presentaba asociación con resistencia a cefixima ($P = ,031$). Los genogrupos (G) mayoritarios fueron el G1407 (13,1%), G2992 (10,3%), G2400 (6,3%) y G387 (3,4%); G1407 y G2400 mostraron asociación con resistencia a ciprofloxacino ($P < ,03$).

Conclusión: Se ha detectado una baja resistencia a ceftriaxona, una preocupante resistencia a azitromicina y una gran variedad de secuenciotipos circulantes, algunos de los cuales presentan correlación con determinados perfiles de resistencia.

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Introduction

Infections caused by *Neisseria gonorrhoeae* (NG) represent a serious public health problem¹. In Europe, the data reported by the European Centre for Disease Prevention and Control (ECDC) point to a marked and progressively increasing incidence². Prompt diagnosis and appropriate treatment combined with preventive and educational measures are fundamental factors in the control of gonococcal infection (GI). In 2012, the main scientific societies started recommending empirical dual therapy of gonorrhoea by combining a third-generation cephalosporin, usually ceftriaxone, with a second-generation macrolide, azithromycin. The appearance in 2018 of the first strain resistant to this combination, along with the change in patterns of susceptibility to antimicrobials, led to the need to reassess the treatment regimen^{3–5}.

Molecular epidemiology is an essential tool for understanding population dynamics⁶, as it enables relationships to be established between local antibiotic susceptibility patterns and circulating strains⁷, which makes it very useful in identifying strains with high resistance and their possible spread among the population⁸. The constant increase in GI cases worldwide, the lack of effective vaccines and the emergence of strains resistant to a wide variety of potential therapeutic options (the World Health Organisation [WHO] recently classified it as a superbug⁹) underline the need to implement surveillance and infection control programmes.

The main aim of the GONovig project was to analyse the antibiotic susceptibility of NG strains isolated in genital samples from patients in the Valencian Community, as well as to carry out molecular typing of the isolates in order to detect epidemiological clusters and their possible association with the resistance profiles detected.

Methods

NG isolates

We conducted a prospective, multicentre study, in which we analysed 227 NG strains obtained from urethral or endocervical swabs taken from patients treated in thirteen representative hospitals in the Valencian Community from January 2013 to December 2018. In order to have a certain geographical representation, a minimum of five isolates were required for each participating centre, corresponding to a single strain per patient and episode. Samples considered to be from sites of colonisation (pharyngeal or anal exudates) were not included in the study, due to the lower prevalence of NG in these biological specimens. Each participating centre identified and carried out the antibiotic susceptibility testing of the isolates using methods available in their laboratory. These centres then sent the original swab and primary culture, refrigerated at 2–8 °C, to Hospital Universitario de La Ribera (HULR), no later than 24–48 h after identifying the isolate, along with demographic data on the patient and the antibiotic susceptibility test carried out

at source. The HULR, a reference centre, re-spread the isolates on Chocolate agar and Thayer-Martin selective medium (bioMérieux, Marcy-l'Étoile, France), and the isolates were re-identified based on the morphology of the colonies, a positive result in the tests for oxidase and catalase, and a high degree of reliability (>95%) in the biochemical gallery using the Vitek-2 system¹⁰ (bioMérieux, Marcy-l'Étoile, France).

Antibiotic susceptibility tests

The isolates collected during the GONovig project were analysed following the international guidelines of the *Clinical and Laboratory Standards Institute* (CLSI)¹¹ and *European Committee on Antimicrobial Susceptibility Testing* (EUCAST)¹². ETEST strips (bioMérieux, Marcy-l'Étoile, France) were used and the Minimum Inhibitory Concentration (MIC) was determined in GC agar, supplemented with 1% IsoVitaleX (BD, New Jersey, United States) and incubated for 20–24 h in the oven at 37 °C with 5% CO₂, against the following antibiotics: penicillin, cefixime, ceftriaxone, azithromycin, ciprofloxacin, spectinomycin and tetracycline. As quality control, the strain NG ATCC 49226 was used.

To minimise possible discrepancies in the reading of the inhibition ellipses, the MIC value was determined by two specialists. The interpretation was carried out comparatively according to CLSI and EUCAST criteria. Additionally, the MIC capable of inhibiting 50% (MIC₅₀) and 90% (MIC₉₀) of the isolates was calculated. We also studied β-Lactamase (penicillinase-producing *Neisseria gonorrhoeae* [PPNG]) production using the cefinase test (BD, New Jersey, USA).

Molecular typing

Molecular characterisation was centralised at the Valencian Community's Fundación para el Fomento de la Investigación Sanitaria y Biomédica (FISABIO) [Foundation for the Promotion of Health and Biomedical Research]. The NG *multi-antigen sequence typing* (NG-MAST) technique was used, based on the sequencing of internal fragments of the hypervariable *porB* and *tbpB* genes, which encode the porin and transferrin of the outer membrane of NG. The QIAamp[®] DNA Mini Kit system (Qiagen, Hilden, Germany) was used. Allele sequences were amplified according to the protocol already described¹³, using a Mastercycler[®] (Eppendorf, Hamburg, Germany) thermal cycler, Master Mix reagents (Biotools, Madrid, Spain) and specific primers (Metabion, Munich, Germany). The allele sequences obtained were entered into the database (www.ng-mast.net) and the resulting sequence types (ST) were recorded. The ST obtained were then grouped into larger associations, called genogroups (G), based on allele similarities¹⁴. To achieve this, the sequences were aligned and associations were established using the MEGA program (<https://www.megasoftware.net>).

Table 1

Percentages of susceptibility to various antibiotics in *Neisseria gonorrhoeae* isolates: comparison according to CLSI and EUCAST criteria.

Antibiotic	CLSI (%)	EUCAST (%)	MIC ₅₀ (mg/l)	MIC ₉₀ (mg/l)
Azithromycin	94	94	0.25	1
Penicillin	88.4	83.8	0.38	1.5
Cefixime	99.5	88	0.016	0.125
Ceftriaxone	98.5	97.6	0.016	0.047
Ciprofloxacin	50.9	46	1	16
Spectinomycin	100	100	–	–
Tetracycline	61.8	49.1	0.75	6

Statistical analysis of the data

Quantitative variables are presented as mean and standard deviation. The Anderson-Darling test was used to verify the normality of the distribution of the quantitative variables, $P < .05$ being considered as statistically significant, with a confidence level of 95%. To establish relationships between qualitative variables, such as antibiotic susceptibility and ST/genogroups, contingency tables were created and Fisher's exact test applied. The statistical calculations were performed with the SPSS Statistics 23 program (IBM, Armonk, United States).

Results

For the strains, 100% were re-identified as NG at the reference centre. Of the 227 isolates analysed, 70% came from hospitals in the city of Valencia, 15% from Alicante and 15% from Castellón. By gender, 216 of the isolates were obtained in males (94%) and 11 in females (6%). The mean age of the population studied was 30.7 (± 9.4) years, with a range from 17 to 79.

Table 1 shows the main results obtained after the antibiotic susceptibility studies. Of the patients with isolates, 6% had MIC > 1 mg/l to azithromycin and were classified as non-wild-type phenotypes carrying mechanisms of resistance¹⁵. The proportion of strains categorised as susceptible to penicillin was 4.1% (both in CLSI and EUCAST), while 84.3% (CLSI) and 79.7% (EUCAST) were of intermediate susceptibility, and 11.6% (CLSI) and 16.2% (EUCAST) were resistant. Of the gonococci, 5.6% showed plasmid resistance to penicillin (PPNG). The proportion of strains resistant to third-generation cephalosporins was 0.5% (CLSI) and 5.9% (EUCAST) for cefixime, and 1.5% (CLSI) and 2.4% (EUCAST) for ceftriaxone. Approximately 50% of the isolates were resistant to ciprofloxacin (CLSI and EUCAST), and 38.2% (CLSI) and 50.9% (EUCAST) were resistant to tetracycline. All the strains analysed were susceptible to spectinomycin.

For technical and budgetary reasons, molecular characterisation was carried out on 175 strains (77% of the total). These typing studies revealed the existence of 86 different ST, made up of 73 different *porB* alleles and 36 different *tbpB* alleles. Nine isolates could not be ST-classified because they corresponded to new sequences or allele combinations not included in the NG-MAST database. The most prevalent ST were ST2992 ($n = 13$), ST3378 ($n = 12$), ST13288 ($n = 12$), ST2400 ($n = 8$) and ST5441 ($n = 6$). The ST detected in ≥ 3 isolates and their alleles are listed in Table 2.

Table 3 shows the four most identified genogroups, along with their ST (primary and secondary) and percentages of resistance associated with antibiotics (according to EUCAST criteria); G1407 (13.1%), followed by G2992 (10.3%), G2400 (6.3%) and G387 (3.4%). The 23 strains included in the main genogroup, G1407, showed resistance to ciprofloxacin ($P > .02$), this genogroup being made up mainly of 13 isolates of ST3378 and only two with ST1407. Two isolates (ST1407 and ST3378) were resistant to cefixime (MIC 0.19 mg/l in both cases) and a third isolate (ST3378) had an MIC > 1 mg/l against azithromycin. One strain belonging to G2992 (ST2992) was resistant to ceftriaxone (MIC 0.75 mg/l). G2400,

Table 2

Main sequence types detected and constituent alleles (*porB* and *tbpB*).

ST	No. of strains	% strains	<i>porB</i> allele	<i>tbpB</i> allele
2992	13	7.4	1,808	29
3378	12	6.9	2,043	110
13288	12	6.9	4,991	10
2400	8	4.6	1,489	563
5441	6	3.4	30	18
21	4	2.3	14	33
387	4	2.3	266	118
437	4	2.3	14	4
4809	4	2.3	2,888	367
5118	4	2.3	3,040	32
11547	4	2.3	6,766	10
292	3	1.7	28	4
5119	3	1.7	3,104	29
8517	3	1.7	1,142	1,531
Others*	91	52	–	–

* Includes 63 different ST.

primarily comprising strains with ST2400, was associated with resistance to ciprofloxacin ($P = .017$) and tetracycline ($P = .048$).

Discussion

It is estimated that more than 376 million new sexually transmitted infections (STI) occur each year, representing a serious public health problem worldwide. GI is the second most prevalent STI of bacterial aetiology, causing a total of 86 million new infections in 2016 alone¹⁶, a significantly higher figure than the 78 million reported by the WHO in 2012. In addition to the increase in its incidence, NG has shown an exceptional capacity to develop mechanisms enabling it to evade the action of many of the antimicrobial agents used for its eradication. The appearance of strains resistant to azithromycin^{15,17} and the changes in the resistance profile of NG have led to modifications to the drug treatment regimen. The current recommendations of the American Centers for Disease Control and Prevention (CDC)³, British Association for Sexual Health and HIV (BSHH)⁵ and European⁴ guidelines propose doubling the dose of ceftriaxone and, in the case of the CDC and BSHH, abandoning the use of azithromycin, establishing ceftriaxone monotherapy as the most recommended empirical therapy.

The 2017 report of the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) highlights Spain as one of the countries with the highest rates of resistance to oral cefixime (5.5%) in the period 2009–2017¹⁸, a finding consistent with our analysis (5.9%). With regards to Spain, in 2011 Pladevall analysed strains isolated in Barcelona that showed resistance rates of 10% to cefixime and 3% to ceftriaxone¹⁹. A subsequent study of the same group with a greater number of strains determined a resistance of 8.3% to cefixime and 2.8% to ceftriaxone²⁰. Cobo et al. reported resistance rates of 6.1% to cefixime and 4.6% to ceftriaxone after analysing samples collected in southern Spain²¹, which decreased to 3.7% (cefixime) and 2.9% (ceftriaxone) in subsequent studies²². The study carried out in Valencia between 2013 and 2019 by Castaño et al. detected 3% of isolates resistant to cefixime and 0.4% to ceftriaxone²³.

Despite the slight geographical and temporal discrepancies, these data confirm NG resistance close to 5% for cefixime and 2% for ceftriaxone according to EUCAST criteria. However, when applying CLSI criteria, these rates are lower, with a particularly evident discrepancy for cefixime (0.5% vs 5.9%), as a result of the different breakpoints established by the two institutions. This can cause problems when classifying the isolates, making it desirable to standardise the cut-off values based on clinical, microbiological and pharmacokinetic criteria.

Table 3
The most prevalent genogroups and percentages of associated antibiotic resistance.

Genogroup (n/%)	Primary ST (n)	Secondary ST (n)	% Azithromycin resistance	% Cefixime resistance	% Ceftriaxone resistance	% Ciprofloxacin resistance	% Tetracycline resistance	% Penicillin resistance
G1407 (23/13%)	1407 (2)	3128 (1) 3149 (2) 3378 (12) 4120 (2) 4936 (1) 5619 (1) 8921 (1) 13123 (1)	4.8	9.5	0.0	100	59.1	95.2
G2992 (18/10%)	2992 (13)	5119 (3) 7636 (2)	0.0	0.0	5.9	0.0	41.2	100
G2400 (11/6,3%)	2400 (8)	9184 (2) 11800 (1)	0.0	0.0	0.0	83.3	100	85.7
G387 (6/3.4%)	387 (4)	1945 (1) 5743 (1)	0.0	0.0	0.0	0.0	16.7	66.7

In the case of azithromycin, an antimicrobial considered the cornerstone in the treatment of several STI, both societies establish an epidemiological cut-off value (ECV) of 1 mg/l, which is the MIC that differentiates wild-type strains^{11,12}. Modern studies suggest that NG has acquired chromosomal mutations in 23S rRNA in recent years¹⁵ which, in conjunction with other resistance mechanisms, enable it to evade the action of macrolides. In Europe, the ECDC²⁴ reported 7.6% resistance to azithromycin in 2018, representing a significant increase compared to the 3.7% in 2017. Resistance to azithromycin detected in other national studies in Spain^{22,23} has been 9% and 9.7%.

The molecular characterisation of the isolates revealed that most (59.3%) of the 86 ST identified were present in a single strain. The main ST detected in our study have also been identified at a European level^{14,25}, with the exception of ST13288, in which three strains with resistance to cefixime were included, alerting us to the possible appearance of a clone, only reported in Italy²⁶, with the potential ability to evade the empirical therapy of choice in GI.

According to the scientific literature, the main genogroup in Spain and Europe is G1407, closely linked to therapeutic failure in France²⁷, Spain²⁸ and the United Kingdom²⁹, as well as other countries. In our analysis, its high prevalence was mainly defined by the 12 strains belonging to ST3378, one of which was resistant to cefixime and another to azithromycin, as well as a statistically significant association with ciprofloxacin-resistant isolates ($P = .02$).

Chisholm et al. performed the molecular typing of 1066 NG strains obtained in 21 European countries participating in the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) from 2009 to 2010¹⁴ concluding that, here in Spain, the most prevalent was G1407, followed by G2992, in line with the results obtained in our study. The ECDC also reported that G1407 was the most common genogroup in Europe in 2009 (23.3%), with its incidence decreasing in 2013 (14.8%)²⁶, followed by genogroups G2992 and G2400, which showed association with strains resistant to ciprofloxacin ($P = .017$) and tetracycline ($P = .048$). The increase in G2400 is a finding also verified by Pladevall, along with its facility for developing antimicrobial resistance mechanisms²⁰. Lastly, in our analysis, G387 had rates similar to those reported in the above European studies^{14,26}.

Establishing links between ST and/or genogroups and specific resistance phenotypes requires molecular typing analyses alongside antibiotic susceptibility testing of the isolates. This highlights the utility of the NG-MAST molecular technique as a complementary method in understanding antimicrobial resistance. Conventional culture continues to be essential to determine the *in vitro* antibiotic susceptibility profile as soon as possible and establish the most appropriate treatment in each case.

One limitation of our study is the fact that the strains analysed belonged only to patients in the Valencian Community. Surveillance, both epidemiological and molecular, is required in areas and countries around us in order to compare results. Another limitation was, despite their lower prevalence, not having included isolates from pharyngeal and anal samples; these have been reported to potentially have different resistance profiles, as the colonising strains in these anatomical areas are subject to less antibiotic pressure and a greater acquisition of resistance genes from other saprophytic species of NG³⁰. Moreover, we did not collect information on the sexual orientation of the patients included in the GONOVig project, and that could be of interest when establishing clinical-epidemiological associations.

In conclusion, the molecular data we obtained show that the circulating NG population in the Valencian Community has similarities with strains isolated in other geographical areas, with ST2992 and ST2400 found to have a particularly high prevalence. Our research shows that there is a correlation between certain ST and/or genogroups and antibiotic resistance, especially to ciprofloxacin and tetracycline. Last of all, resistance to third-generation cephalosporins remained low, particularly for ceftriaxone (2.4%) compared to cefixime (5.9%), while strains with high MIC of azithromycin have increased in recent years (6%).

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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