European Journal of Clinical Microbiology & Infectious Diseases (2024) 43:1009–1012
https://doi.org/10.1007/s10096-024-04786-y

BRIEF REPORT

Comparison of gradient diffusion and molecular methods using Allplex™ NG&DR assay (Seegene®) for macrolide and fluoroquinolone screening resistance in *Neisseria gonorrhoeae*

Alfredo Maldonado-Barrueco[1] · Claudia Sanz-González[1] · Iker Falces-Romero[1,2] · Paloma García-Clemente[1] · Juana Cacho-Calvo[1] · Inmaculada Quiles-Melero[1]

Received: 22 December 2023 / Accepted: 12 February 2024 / Published online: 26 February 2024

Abstract

Antimicrobial resistance in *Neisseria gonorrhoeae* (NG) is increasing worldwide. Second-line treatments with macrolides or fluoroquinolones are an option for NG infections in some cases following the STI guideline recommendations. In our study, we compared the gradient diffusion test using EUCAST 2024 breakpoints with a new molecular method using the Allplex™ NG&DR assay (Seegene®) including A2059G/C2611 mutations (23S rRNA) associated with high/moderate-level macrolide resistance and S91F mutation (*gyrA*) relationship with fluoroquinolone resistance in NG isolates ($n=100$). We calculated the sensitivity, specificity, and correlation of the molecular test for fluoroquinolone using the gradient diffusion as the reference method. In twenty-three strains was not detected any mutation associated with macrolides or fluoroquinolone resistance. No A2059G/C2611T mutations were detected, and the S91F mutations were detected in 77 out of the 100 isolates screened. Twenty-three NG isolates were reported to be resistant to azithromycin (ECOFF: >1 mg/L), and 78 NG isolates were resistant to ciprofloxacin (MIC: >0.06 mg/L). The molecular method showed a sensitivity of 96.1% and, a specificity of 90.9% for fluoroquinolone susceptibility, but the statistical analysis between the molecular test and gradient diffusion test was not statistically significant for fluoroquinolone resistance ($p=1$). Statistical analysis was not performed for macrolides because of the absence of positive RT-PCR results. According to our data, Allplex™ assay cannot replace the gradient diffusion test for macrolide resistance. However, the assay could be used to test fluoroquinolone resistance in NG isolates as a replacement for phenotypic methods.

Keywords Neisseria gonorrhoeae · Azithromycin · Ciprofloxacin · Resistance · Mutations · gyrA · ParC · Macrolide · Fluoroquinolone · Seegene · PCR

Introduction

Antimicrobial resistance in *Neisseria gonorrhoeae* (NG) is increasing worldwide [1]. The mechanisms of NG resistance to macrolides include chromosomal mutations, genes encoding rRNA methyltransferases, and overexpression of efflux pumps [2]. Resistance to fluoroquinolones is mainly due to the presence of mutations in *gyrA* or *parC* genes, among others [3]. The first-line treatment for NG infections is the third-generation cephalosporins, such as intramuscular ceftriaxone or oral cefixime [4]. However, macrolides (azithromycin) are second-line treatments that should not be used as monotherapy for NG infections [4]. Treatment with fluoroquinolones (ciprofloxacin) is an option in patients allergic or intolerant to third-generation cephalosporins, provided that a *gyrA* mutation is not detected prior to fluoroquinolone therapy following the sexually transmitted infections (STIs) guidelines [4, 5].

New commercial assays are emerging for the detection of mutations associated with therapeutic failure of these

Alfredo Maldonado-Barrueco and Claudia Sanz-González contributed equally to this work.

✉ Alfredo Maldonado-Barrueco
alfredo.maldonado@salud.madrid.org

[1] Clinical Microbiology Department, Hospital Universitario La Paz, 261, Madrid 28046, Spain

[2] CIBERINFEC, Instituto de Salud Carlos III, Madrid, Spain

second-line treatments by nucleic acid amplification testing in NG infections [6]. Previously, between October 2022 and January 2023, our working STI group reported the prevalence of mutations associated with macrolide and fluoroquinolone resistance in NG from direct sampling in our population using one of these commercially available CE kits (Allplex™ NG&DR Assay, Seegene®). We reported a prevalence of 0.9% mutations associated with macrolide resistance, and 60% of mutations associated with fluoroquinolone resistance at the Hospital Universitario La Paz (HULP) [7]. However, the molecular-level genotypic detection of resistance associated with mutations could not be expressed phenotypically by diffusion gradient methods [8]. The aim of our study was to test the association between gradient diffusion and Allplex™ assay methods for the antimicrobial susceptibility to macrolides and fluoroquinolones in NG isolates.

Methods

In a prospective observational cohort study design, we collected the cultures of NG samples (previously confirmed by real-time PCR, RT-PCR) from February to May 2023 for antimicrobial susceptibility phenotyping and genotyping testing at HULP. Samples including urethral, oropharyngeal, endocervical, and rectal swabs (Deltalab®, Barcelona, Spain) were screened during the study period for NG using RT-PCR with Allplex™ 7 STI Essential Assay (Seegene®, Seoul, South Korea), which includes *Neisseria gonorrhoeae*, among other STI pathogens. First-void urine samples were excluded from the study because of the difficulty in culturing NG isolates in this type of sample. Only one NG strain from each patient was included in the study.

Phenotypic method: Culture of *Neisseria gonorrhoeae isolates*

Samples were collected of patients with Citoswab® amies medium (Citotest labware manufacturing Co., LTD, Halmen City, China). Swabs were cultured in chocolate and chocolate agar PolyVitex mediums (BioMérieux®, Marcy l´Etoile, France) and grown for 48 h with 5% CO_2 atmosphere. Following the EUCAST Clinical Breakpoint Tables v. 14.0 (2024), disk diffusion criteria for antimicrobial susceptibility testing of NG have not yet been defined, and an MIC method should be used [9]. Antimicrobial susceptibility NG testing for azithromycin and ciprofloxacin was performed by gradient diffusion (0.5 McFarland) using MIC Test Strip™ (Liofilchem®, Roseto degli Abruzzi, Italy) following EUCAST 2024 breakpoints: isolates with an ECOFF (epidemiological cut-off) >1 mg/L breakpoint for azithromycin testing, and MIC >0.06 mg/L for ciprofloxacin were considered resistant [9].

Antimicrobial resistance testing with 0.5 McFarland and MIC Test Strip™ was performed using chocolate agar medium (BioMérieux®) with growing during 24 h by 5% CO_2 atmosphere. MICs interpreted as "susceptible, increased exposure" (I category, 0.06 mg/L) were considered as susceptible strains. The gradient diffusion method was used as the reference test for the antimicrobial susceptibility of macrolides and fluoroquinolones. Culti-Loops™ *Neisseria gonorrhoeae* ATCC™ 19,424™ controls were used as standards for the phenotypic method.

Genotypic method: RT-PCR of *Neisseria gonorrhoeae* isolates

The previous NG isolates studied by phenotypic methods were analysed by RT-PCR test. Direct samples were not screened using RT-PCR to avoid false positive resistance by another *Neisseria* spp. in the samples. The DNA extraction was performed using a MagCore Super instrument extractor (RBC Bioscience Corp®, New Taipei City, Taiwan) according to the manufacturer's instructions. Molecular analysis was performed using the kit Allplex™ NG&DR Assay (Seegene®) to detect mutations associated with macrolides and fluoroquinolone resistance. This is a new commercial CE assay authorised for in vitro diagnostic use. The assay includes the A2059G mutation (23S rRNA) associated with high-level macrolide resistance and the C1126T mutation (23S rRNA) associated with moderate-level macrolide resistance. Resistance associated with fluoroquinolones was detected by the S91F (*gyrA*) mutation in the assay. The NG extracts were amplified using CFX96™ Touch Real-Time PCR Detection System thermal cycler (Bio-Rad®, Hercules, California). Internal, positive, and negative controls were used for each RT-PCR run.

We calculated the sensitivity, specificity, and correlation between the Allplex™ commercial assay and the gradient diffusion method using the *McNemar* test. A p value of less than 0.05 ($p < 0.05$) was considered statistically significant. Statistical analysis was not performed for macrolides because of the lack of positive RT-PCR results.

Results

A total of one hundred NG isolates were screened for macrolides and fluoroquinolone resistance using molecular and gradient diffusion methods during the study period. According to the molecular assay results, in twenty-three strains (23%) no was detected mutations associated with macrolide or fluoroquinolone resistance. Moreover, any C1126T

or A2059G mutations (23S rRNA) associated with moderate/high-level macrolide resistance were detected in the NG isolates screened. On the other hand, in 77 out of 100 NG strains (77%) analysed, the S91F mutation was detected, implying fluoroquinolone resistance.

According to the gradient diffusion test results and following EUCAST breakpoints, 23 NG isolates (23%, MIC > 1 mg/L) were reported as resistant to azithromycin, and 78 NG isolates (78%, MIC > 0.06 mg/L) were reported as resistant to ciprofloxacin out of the 100 strains studied. However, in three out of 78 NG isolates (3.85%), no mutation relationship with fluoroquinolones was detected. However, eighteen NG isolates (18%) were reported to be resistant to macrolides and fluoroquinolones using the gradient diffusion test.

The sensitivity, specificity, and correlation of the Allplex™ assay test for macrolides could not be determined because of the absence of A2059G/C1126T mutations. The assay showed a sensitivity of 96.1%, and a specificity of 90.9% for fluoroquinolone susceptibility. Although the phenotypic method showed a 97.4% correlation with the genotypic test for fluoroquinolone, the statistical study correlation between methods was considered not statistically significant ($p = 1$) for fluoroquinolone (ciprofloxacin) resistance screening.

Discussion

Antimicrobial resistance in NG is increasing wordwide [10]. Previously, we reported the prevalence of mutations associated with macrolide and fluoroquinolone resistance in the HULP population with NG infections. In the present study, we report the correlation between genotypic and phenotypic methods for macrolide and fluoroquinolone resistance using the new Allplex™ NG&DR (Seegene®) assay.

The therapeutic use of macrolides (azithromycin) and fluoroquinolones (ciprofloxacin) represents a useful second-line therapy for NG infections [4]. Sánchez-Busó et al. reported an overall prevalence of 8% macrolide resistance in Europe [10]. In Spain, NG azithromycin susceptibility studies have reported a prevalence between of 2–12% using the gradient diffusion methods [11–13]. The absence of the A2059G/C1126T mutations associated with high/moderate macrolide resistance in our study was in consistent with other reports [2]. However, the phenotypic method reported more azythromicin resistant isolates than Allplex™ genotypic method. These results could be explained by the fact that the Allplex™ assay includes only two macrolide resistance targets. Kandinov et al. reported that the overexpression of efflux pumps by mutations in the MtrCDE genes caused more than 90% of azithromycin resistance in NG [2]. Furthermore, other less prevalent NG mutations, such as A2058G or resistances associated with overexpression of the efflux system, are associated with macrolide resistance in NG [2]. This could justify the discordance between the methods. In our study, the 23% of NG isolates were discordant between methods favouring to the phenotypic method. The Allplex™ assay could not replace the phenotypic method for NG azithromycin resistance screening.

Fluoroquinolone therapy is another second-line treatment for NG infections. High prevalence of fluoroquinolone resistance in Europe [10]. The STI guidelines recommend the screening of mutations in the *gyrA* gene prior to treatment with fluoroquinolones in NG infections [4]. We detected using the Allplex™ assay a high prevalence of S91F mutation associated with failure to receive fluoroquinolone treatment, although this correlation was not statistically significant. These results could be explained due to other mutations less prevalent associated with fluoroquinolone resistance, such as D95A, D95G, D95N (*gyrA*), or D86N (*parC*), among others are not included in Allplex™ assay [3]. However, compared with macrolide resistance, fluoroquinolone resistance is mainly related to the S91F mutation in NG. Therefore, the Allplex™ assay could replace the phenotypic method (gradient diffusion) for the detection of fluoroquinolone resistance prior to the treatment with ciprofloxacin in NG infection.

As a limitation to our study, Sanger or whole-genome sequencing analysis was not performed to identify the resistance mechanisms not detected by the Allplex™ assay or discordant results between phenotypic and genotypic methods for macrolide and fluoroquinolone resistance.

In summary, the phenotypic method based on gradient diffusion can be used as a reference method for the study of MIC antimicrobial susceptibility [8, 9]. However, the phenotypic method requires the culture of NG samples. Nevertheless, the delay in seeding the samples would prevent the strain from being obtaining due to the lability of the NG strain. New commercial molecular methods allow the rapid genotypic detection of resistance-associated mutations. Although these techniques require the accumulation of several samples in order to be cost-effective, their use could reduce the time to laboratory response and allows the resistance detection in direct samples such as endocervical, urethral, or rectal samples without the need for culturing. Further studies are necessary for evaluating the Allplex™ NG&DR Assay (Seegene®) assay in direct samples. However, the Allplex™ assay could not be sufficient for screening for azithromycin resistance, requiring the use of gradient diffusion tests, but could be used for testing ciprofloxacin resistance in NG.

Acknowledgements To my mentor and friend, Inmaculada Quiles Melero, for trusting me during these years and supporting me in my

personal and professional life.

Author contributions AMB, CSG: design, analysis of data, and writing of the manuscript. IFR: serological data, review of the manuscript. PGC: design, review of the manuscript. JCC: review of the manuscript. IQM: design, writing of the manuscript, and review of the manuscript.

Funding Not funding.

Declarations

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The data used are from clinical samples that were necessary for the clinical STI diagnosis of the patients. The detection of resistance associated with macrolide and fluoroquinolone in *Neisseria gonorrhoeae* was not incorporated into the medical clinical reports of the patients.

References

1. Unemo M, Lahra MM, Escher M, Eremin S, Cole MJ, Galarza P et al (2021) WHO global antimicrobial resistance surveillance for *Neisseria gonorrhoeae* 2017–18: a retrospective observational study. Lancet Microbe 2(11):e627–e636. https://doi.org/10.1016/S2666-5247(21)00171-3
2. Kandinov Ilya S, Boris K, Dmitry V, Alexandra G, Sofya K, Alexey et al (2023) Azithromycin susceptibility testing and molecular investigation of *Neisseria gonorrhoeae* isolates collected in Russia, 2020–2021. Antibiotics 12(1):170. https://doi.org/10.3390/antibiotics12010170
3. Beata M-B, Cezary K, Aneta K-U, Marusza Wojciech (2022) Molecular mechanisms of drug resistance and epidemiology of multidrug-resistant variants of *Neisseria gonorrhoeae*. Int J Mol Sci 23(18):10499. https://doi.org/10.3390/ijms231810499
4. Workowski KA, Bachmann LH, Chan PA, Johnston CM, Muzny CA, Park I, Reno H, Zenilman JM, Bolan GA (2021) Sexually transmitted infections treatment guidelines, 2021. MMWR Recomm Rep 70(4):1–187. https://doi.org/10.15585/mmwr.rr7004a1
5. Helen F, John S, Suneeta S, Tariq SS, FitzGerald Mark (2018) UK national guideline for the management of infection with *Neisseria gonorrhoeae*. Int J STD AIDS 31(1):4–15. https://doi.org/10.1177/0956462419886775
6. Sánchez NO, Pérez NF, Martínez SB (2022) Evaluation of the viasure *Neisseria gonorrhoeae* ciprofloxacin resistant assay for the simultaneous identification and direct detection of ciprofloxacin susceptibility. Diagn Microbiol Infect Dis 104(4):115798. https://doi.org/10.1016/j.diagmicrobio.2022.115798
7. Maldonado-Barrueco A, Sanz-González C, Falces-Romero I, García-Clemente P, Cacho-Calvo J, Quiles-Melero I (2023) Prevalence of mutations associated with macrolide and fluoroquinolone resistance in *Neisseria gonorrhoeae* with AllplexTM NG&DR Assay (Seegene®) in a tertiary hospital from Madrid, Spain. Rev Esp Quimioter. https://doi.org/10.37201/req/058.2023
8. Qi C, Stratton CW, Zheng X (2006) Phenotypic testing of bacterial antimicrobial susceptibility. In: Advanced techniques in diagnostic microbiology. Springer, Boston. https://doi.org/10.1007/0-387-32892-0_5
9. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 14.0, (2024) http://www.eucast.org.n.d
10. Sánchez-Busó L, Cole MJ, Spiteri G, Day M, Jacobsson S, Golparian D et al (2022) Europe-wide expansion and eradication of multidrug-resistant *Neisseria gonorrhoeae* lineages: a genomic surveillance study. Lancet Microbe 3(6):e452–e463. https://doi.org/10.1016/S2666-5247(22)00044-1
11. Cobo F, Cabezas-Fernández MªT, Cabeza-Barrera MªI (2016) Antimicrobial susceptibility and typing of *Neisseria gonorrhoeae* strains from Southern Spain, 2012–2014. Enfermedades Infecciosas y microbiología clínica 34(1):3–7. https://doi.org/10.1016/j.eimc.2015.01.017
12. Guerrero-Torres MD, Menéndez MB, Guerras CS, Tello E, Ballesteros J, Clavo P et al (2019) Epidemiology, molecular characterisation and antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates in Madrid, Spain, in 2016. Epidemiol Infect 147:e274. https://doi.org/10.1017/S095026881900150X
13. García UI, Nieto Toboso MC, Azpeitia EM, Imaz Perez M, Hernandez Ragpa L, Álava Menica JA et al (2020) Epidemiological surveillance study of gonococcal infection in Northern Spain. Enfermedades Infecciosas y Microbiología Clínica ;38(2):59–64. https://doi.org/10.1016/j.eimc.2019.05.002