Journal of Global Antimicrobial Resistance 50 (2026) 91–94

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

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

Genome Note

Genomic characterisation and global phylogenomic placement of a high-level azithromycin (HL-AziR) and fluoroquinolone-resistant *Neisseria gonorrhoeae* ST9363 strain from Chile

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ARTICLE INFO

Article history:
Received 8 January 2026
Revised 15 May 2026
Accepted 16 June 2026
Available online 24 June 2026

Editor: Xi Li

Keywords:
Neisseria gonorrhoeae
ST9363
Fluoroquinolone-resistance
High-level azithromycin resistance
WHO priority pathogen

ABSTRACT

Background: Antibiotic-resistant *Neisseria gonorrhoeae* (Ng) is a WHO priority pathogen and represents a serious public health threat. This study reports the first Chilean Ng ST9363 isolate (GC11/23) exhibiting dual high-level resistance to azithromycin and fluoroquinolone, recovered from a urethral discharge sample in July 2023.

Methods: Antimicrobial susceptibility was assessed using disk diffusion and MIC strip tests. Whole-genome sequencing (WGS) was performed using a hybrid approach, followed by annotation using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). *In silico* analyses included Staramr, MLST and NG-STAR. Phylogenetic analysis was performed on the core genome of 2417 global ST9363 Ng isolates using IQ-TREE and visualised with TreeViewer.

Results: GC11/23 exhibited resistance to ciprofloxacin, tetracycline, penicillin and azithromycin, while remaining susceptible only to ceftriaxone and spectinomycin. The isolate belonged to ST9363 and NG-STAR 3194. Mutations associated with fluoroquinolone resistance were identified in *gyrA* and *parC*, while high-level azithromycin resistance (HL-AziR) was linked to mutations in all four copies of the *23S rRNA* gene, as well as in *mtrD* and *mtrR*. Additional resistance-associated alterations included an insertion (ins346D) in *penA* and a mutation in *rpsJ*, conferring resistance to penicillin and tetracycline, respectively. Phylogenetic analysis clustered GC11/23 with isolates from the USA and Europe collected in 2019.

Conclusions: GC11/23 represents a multidrug-resistant Ng strain belonging to ST9363/NG-STAR 3194 and carrying mutations conferring resistance to fluoroquinolones, azithromycin, penicillin and tetracycline. Its phylogenetic placement within a European lineage highlights the global dissemination of HL-AziR and fluoroquinolone-resistant strains.

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https://doi.org/10.1016/j.jgar.2026.06.008

Introduction

Neisseria gonorrhoeae (Ng) is a strict human pathogen of increasing global concern due to its rising incidence and remarkable capacity to develop resistance to multiple antimicrobial classes, including macrolides and fluoroquinolones. In 2024, the World Health Organization (WHO) classified Ng strains resistant to third-generation cephalosporins and/or fluoroquinolones as a high-priority threat because of their significant public health impact [1]. Furthermore, azithromycin remains part of combination therapy in several international treatment guidelines [1], making the investigation of high-level azithromycin-resistant (HL-AziR) isolates particularly important.

In this context, the Ng ST9363 clone has emerged as particularly significant owing to its worldwide dissemination and its consistent association with azithromycin resistance [2], making it a critical target for surveillance. Continuous molecular monitoring is therefore essential to elucidate the dissemination dynamics of such high-risk clones, particularly in geographic regions where the molecular epidemiology of Ng remains poorly characterised, such as Latin America [2]. In this context, we report the first Chilean Ng strain (GC11/23) exhibiting dual high-level azithromycin (HL-AziR) and fluoroquinolone resistance.

Materials and methods

The Ng isolate GC11/23 was collected in July 2023 from a urethral discharge specimen obtained from an 18-year-old male patient at a hospital in Concepción, Chile and was initially identified using MALDI-TOF/MS. Antimicrobial susceptibility testing was performed using disk diffusion according to CLSI (M-100, 2024) guidelines, and MICs for azithromycin and ceftriaxone were determined using gradient diffusion strips (Liofilchem, Italy®).

Genomic DNA was sequenced using short- and long-reads utilising an Illumina NovaSeq X Plus platform and a MinION Mk1B device equipped with an R10.4.1 (FLO-MIN114) flow cell (Oxford

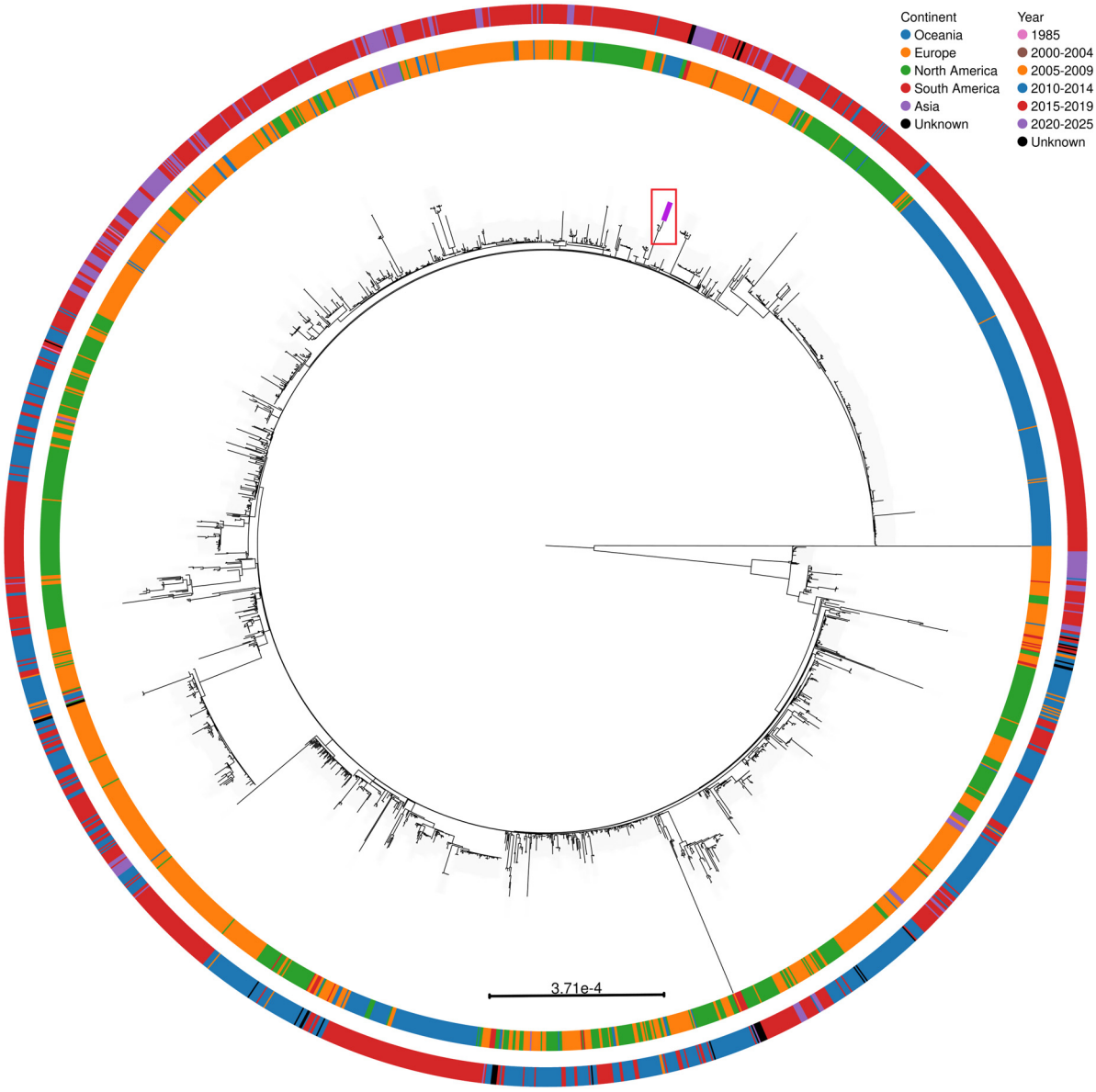

Fig. 1. A recombination-free core genome phylogeny of ST9363 *Neisseria gonorrhoea* isolates. The isolate of interest, GC11/23, is shown with a purple rectangle on the tip. Scale bar indicates nucleotide substitutions per site. Isolation continent and year range is shown with coloured bars as indicated by the legend, with the inner ring showing continent and the outer ring showing year range. The red rectangle highlights the zoomed-in clade that is further analysed in panel 1B.

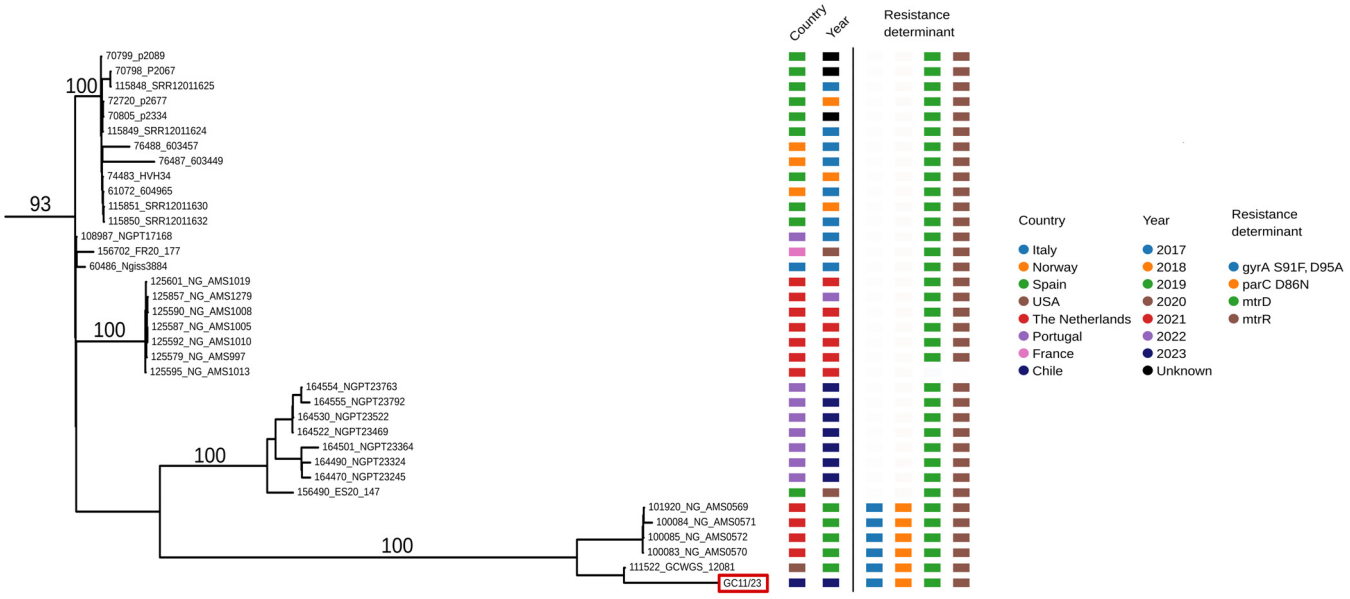

Fig. 2. A subtree showing isolate GC11/23 among closely related strains. Support values were calculated from 1000 bootstrap replicates, with relevant values shown on the corresponding branch.

Nanopore Technologies, Cambridge, UK), and a hybrid assembly was generated with Unicycler (v0.5.1) and annotated using the NCBI PGAP pipeline (v6.7). Species identification was carried out by fastANI (v1.3), resistance genes were predicted with AMRFinderPlus (v1.0.20) and point mutations were detected using staramr (v0.11.0) with the PointFinder available at the Galaxy Australia server [3]. Multilocus sequence typing (MLST) typing was performed with mlst (v2.22.0) [3], while NG-STAR and NG-MAST profiles were assigned using PathogenWatch (https://pathogen.watch/).

For phylogenomic analyses, ST9363 genomes were retrieved from PubMLST (accessed 9 September 2025) and quality filtered using CheckM (v1.2.4), resulting in 2417 genomes. Sequence typing and functional annotation were performed, followed by coregenome alignment generated with Panaroo (v1.5.2). Phylogenetic inference was conducted using IQ-TREE (v2.4.0) with model selection, and recombination was detected and masked prior to reconstruction. The final recombination-masked phylogeny was reconstructed with bootstrap support (1000 bootstrap replicates), pruned, midpoint-rooted and visualised with TreeViewer.

Results and discussion

The GC11/23 isolate was confirmed as *N. gonorrhoeae* by fastANI (>95% average nucleotide identity). Phenotypic testing revealed resistance to ciprofloxacin, tetracycline, penicillin and azithromycin (MIC > 256 µg/mL), while susceptibility to ceftriaxone (MIC < 0.016 µg/mL) and spectinomycin was retained, classifying GC11/23 as a high–level azithromycin–resistant (HL–AziR) strain.

The genome of GC11/23 had a total length of 2 174 808 bp assembled into 2 contigs with an overall G+C content of 52.57%. The N50 and L50 values were 2 170 601 and 1, respectively. Furthermore, a total of 2173 coding-DNA sequences (CDS), 12 rRNA, 56 tRNA and 1 tmRNA sequences were identified. The chromosome was composed by a contig of 2 170 601 bp, whereas a contig of 4207 bp was also detected, which matches (>99% identity) with a cryptic plasmid identified in a Ng isolate collected in Thailand in 2010 (accession number CP048932.1).

The resistome of GC11/23 comprises diverse genetic determinants mediating resistance to clinically relevant antibiotics. GC11/23 harboured the D346ins in the *penA* gene, which confers resistance to penicillin, confirming a nonmosaic *penA* allele. This is particularly relevant, as mosaic *penA* alleles are associated with decreased susceptibility or resistance to ceftriaxone [4]; thus, this finding explains the ceftriaxone susceptibility of GC11/23. In addition, fluoroquinolone resistance in GC11/23 was associated with the canonical mutations S91F and D95A in GyrA, and D86N in ParC. These mutations compromise the efficacy of ciprofloxacin therapy and may be horizontally transferred via transformation [5], a process facilitated by the natural competence of Ng. Also, GC11/23 was susceptible to spectinomycin, being active against this isolate as the primary resistance mechanism – the C1192T substitution in the *16S rRNA* genes [6] – was absent in GC11/23. In contrast, this strain was resistant to tetracycline as it presented the V57M substitution in *rpsJ*, a widely distributed mutation among WHO reference *Ng* strains [7]. Its widespread presence among these reference strains highlights its role as a baseline resistance marker [7], often acting synergistically with other chromosomal determinants such as *mtrR* [7]. For azithromycin, GC11/23 contained the A2045G mutation in all four copies of the *23S rRNA* gene (Figs. 1 and 2), a well-established molecular determinant responsible for the majority of globally reported HL-AziR. Additionally, this strain carried the *mtrD/mtrR* promoter mosaic 2, which further contributes to macrolide resistance [8]. Isolates harbouring these mutations were detected in the European Economic Area (EEA) in 2020, the USA and in Argentina [8], indicating international dissemination of this resistant genotype.

Molecular typing showed that GC11/23 belongs to MLST ST9363 and NG-STAR ST3194, and a novel NG-MAST type as it presents a new *porB* allele. Notably, ST9363 has been associated to global dissemination and reduced susceptibility to azithromycin, primarily driven by mutations in the *mtrCDE* efflux operon [2]. Phylogenetic analysis of the 2417 ST9363 Ng genomes analysed demonstrated that GC11/23 clustered within a well-supported clade (bootstrap value = 100) (Fig. 1) alongside isolates from the USA and the Netherlands, collected in 2019 (Fig. 2). This lineage is part of a broader cluster that includes strains from multiple countries (Italy, Norway, Spain, USA, Portugal, France and Chile) sampled from 2017 to 2023, indicating extensive international dissemination (Fig. 2). The high bootstrap support across major nodes underscores the

genetic relatedness of these isolates and suggests sustained global transmission of this lineage.

Across the global ST9363 dataset, resistance–associated mutations showed distinct phylogenetic patterns. High–level azithromycin resistance determinants, including the *23S rRNA* A2045G mutation and *mtrR/mtrD* alterations, were largely confined to specific well–supported subclades, including the lineage containing GC11/23 (Figs. 1 and 2). In contrast, fluoroquinolone resistance mutations in *gyrA* (S91F, D95A) and *parC* (D86N) displayed a broader and less structured phylogenetic distribution, suggesting multiple acquisition events or earlier emergence and distinct evolutionary trajectories of antimicrobial resistance within ST9363. Although GC11/23 remained susceptible to ceftriaxone due to a nonmosaic *penA* allele, the ST9363 lineage is considered 'high risk' because of its capacity to acquire additional ceftriaxone resistance determinants, including mosaic *penA* alleles or mutations in *ponA*.

In conclusion, GC11/23 represents a HL-AziR Ng isolate harboring multiple resistance-associated mutations and belonging to a globally disseminated lineage, providing the first genomic evidence of internationally circulating ST9363 strains in Chile and underscoring the importance of continued genomic surveillance to inform treatment strategies and monitor the emergence of resistance.

Data availability: This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JBFSHC000000000. The version described in this paper is version JBFSHC020000000.

Funding: This work was supported by the VRID-UDEC Interdisciplinario N° 2022000671INT awarded to F. M-L, by the Pfizer Grant Nr 60340625 awarded to G. G-R and by the Fondo de Apoyo a la Investigación (FAI) – 2023 of the Faculty of Medicine of Universidad de Concepción awarded to A. A.-R.

Ethical approval: This study was approved by the Scientific Ethical Committee of the Concepción Health Service, Concepción, Chile (approval date: 23 January 2024; Project ID: CEC 23-12-72).

Declaration of generative AI and AI-assisted technologies in the writing process: During the preparation of this work the author(s) used Copilot in order to improve the grammar of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of competing interest: None declared.

Acknowledgements: The authors would like to thank the Laboratorio de Microbiología of the Hospital Regional Guillermo Grant Benavente in Concepcion for providing the GC11/23 isolate.

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