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Genomic characterization of invasive *Neisseria meningitidis* in Spain (2011/12–2022/23): expansion of clonal complex 213 and the potential threat to 4CMenB vaccine strain coverage

Josep Roca-Grande [a,b,c], Alba Mir-Cros [a,c], Carmen Muñoz-Almagro [d,e,f], Mayli Lung [g], Alba Bellés-Bellés [h], Jordi Càmara [i,j], Emilia Cercenado [j,k], M. Ángeles Galán-Ladero[l], Andrea Martín-Nalda [m], Albert Moreno-Mingorance [a,c], Daniel Navarro de la Cruz [n], M. Ángeles Orellana[o], Begoña Palop[p], Amaresh Pérez-Argüello[d], Guillem Puigsech-Boixeda [a,b,c], M. Dolores Quesada [q], Alba Rivera [b,r], Ana Rodriguez-Fernandez[s], Enrique Ruiz de Gopegui [t], Carolina Sarvisé[u], Aleix Soler-Garcia [d,v], Belén Viñado [g], Nieves Larrosa [a,b,c,g], Juan José González-López [a,b,c,g] and the GE-EMIE Study Team*

[a]Microbiology Research Group, Vall d'Hebron Institut de Recerca (VHIR), Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain; [b]Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Bellaterra, Spain; [c]CIBER de Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III, Madrid, Spain; [d]Infectious Diseases and Microbiome Research Group, Institut de Recerca Sant Joan de Déu, Hospital Sant Joan de Déu, Esplugues, Spain; [e]CIBER de Epidemiología y Salud Pública (CIBERESP), Instituto de Salud Carlos III, Madrid, Spain; [f]School of Medicine and Health Sciences, Universitat Internacional de Catalunya, Barcelona, Spain; [g]Department of Clinical Microbiology, Hospital Universitari Vall d'Hebron, Barcelona, Spain; [h]Department of Clinical Microbiology, Hospital Universitari Arnau de Vilanova, Institut de Recerca Biomèdica de Lleida (IRB-Lleida), Lleida, Spain; [i]Department of Clinical Microbiology, Hospital Universitari de Bellvitge, IDIBELL-UB, L'Hospitalet de Llobregat, Spain; [j]CIBER de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III, Madrid, Spain; [k]Department of Clinical Microbiology and Infectious Disease, Hospital General Universitario Gregorio Marañón, Madrid, Spain; [l]Department of Clinical Microbiology, Hospital Universitario Reina Sofía, Córdoba, Spain; [m]Pediatric Infectious Diseases and Immunodeficiencies Unit, Children's Hospital, Hospital Universitari Vall d'Hebron Campus, Barcelona, Spain; [n]Department of Clinical Microbiology, Complejo Hospitalario Universitario de Santiago, Santiago de Compostela, Spain; [o]Department of Clinical Microbiology, Hospital Universitario 12 de Octubre, Madrid, Spain; [p]Department of Clinical Microbiology, Hospital Regional Universitario de Málaga, Málaga, Spain; [q]Department of Clinical Microbiology, Hospital Universitari Germans Trias i Pujol, UAB, Badalona, Spain; [r]Department of Clinical Microbiology, Hospital de la Santa Creu i Sant Pau, Sant Pau Biomedical Research Institute (IIB Sant Pau), Barcelona, Spain; [s]Department of Clinical Microbiology, Hospital Universitario Marqués de Valdecilla-IDIVAL, Santander, Spain; [t]Department of Clinical Microbiology, Hospital Universitario Son Espases, Instituto de Investigación Sanitaria Illes Balears (IdISBa), Palma de Mallorca, Spain; [u]Department of Clinical Microbiology, Hospital Universitari de Tarragona Joan XXIII, Pere Virgili Health Research Institute (IISPV), Tarragona, Spain; [v]Department of Pediatrics, Hospital Sant Joan de Déu, Esplugues, Spain

ABSTRACT

Invasive meningococcal disease (IMD) is associated with significant global morbidity and mortality and is addressed by conjugated polysaccharide and subcapsular vaccines. In Spain, data on 4CMenB vaccine strain coverage and antimicrobial susceptibility are limited. This study aimed to describe the genomic epidemiology, predict 4CMenB vaccine strain coverage, and assess antimicrobial susceptibility of 323 *Neisseria meningitidis* isolates causing IMD, collected from 57 Clinical Microbiology Laboratories in Spain over 12 years (2011/12–2022/23). Whole genome sequencing was performed to identify serogroup, clonal complex (cc), and antimicrobial resistance determinants. Vaccine strain coverage for serogroup B (MenB) isolates was predicted using the genetic Meningococcal Antigen Typing System approach. The most prevalent serogroups were B (57.9%), W (21.4%), C (10.4%), and Y (8.4%). MenB predominated throughout most seasons, except during the 2019/20 season when serogroup W peaked. Post-COVID-19 pandemic, MenB remained the most frequent (70.2%). Thirteen cc were identified among MenB isolates, with cc213 being the most prevalent (40.1%). Only 28.9% of MenB isolates were predicted to be covered by 4CMenB, with cc213 showing an exceptionally low coverage rate (5.3%) due to antigenic variants poorly targeted by the vaccine. Notably, cc213 was responsible for twice the proportion of MenB cases in 4CMenB-vaccinated versus unvaccinated. All isolates were susceptible to third generation cephalosporins, and 13.5% showed penicillin resistance. This study highlights the alarming prevalence of cc213 among MenB IMD cases in Spain and the limited 4CMenB coverage against this cc. The disproportionate representation of cc213 in vaccinated individuals underscores its potential to compromise vaccine effectiveness.

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KEYWORDS *Neisseria meningitidis*; whole genome sequencing; genomic epidemiology; cc213; gMATS; 4CMenB vaccine strain coverage; antimicrobial susceptibility

CONTACT Juan José González-López juanjo.gonzalez@vallhebron.cat
*Study Group Team Members are Listed in Acknowledgments.
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Introduction

Neisseria meningitidis is responsible for invasive meningococcal disease (IMD), which is associated with high morbidity and mortality. In the European Economic Area (EEA), the case fatality rate is approximately 8–15%, depending on age [1]. The highest IMD incidence occurs in infants and children under five, especially those under one year of age, although older adults and individuals with some primary and secondary immunodeficiencies are also at risk [1–4]. Standard antibiotic treatment for IMD involves β-lactams, with third-generation cephalosporins (3GC) preferred empirically, and rifampicin or ciprofloxacin for prophylaxis in close contacts [5]. Resistance to penicillin is an emerging concern, with resistance rates in Europe ranging from 0.7% to 15.9% [6–8], involving mutations in the penicillin-binding protein (PBP2), encoded by the *penA* gene. Recently, *N. meningitidis* carrying the ROB-1 β-lactamase conferring penicillin resistance has been reported [9,10]. Resistance to rifampicin and ciprofloxacin is rare in Europe [6–8], while Japan and China show higher rates of ciprofloxacin resistance (7.9% and 67.7%, respectively), usually linked to mutations in the *gyrA* and *parC* genes [11,12].

Globally, serogroups A, B, C, W, X, and Y account for most IMD cases [13]. In the EEA, serogroup B is the leading cause of IMD, accounting for 51% of cases in 2018 [2]. However, an increase of more than 500% and 130% in the incidence of serogroups W and Y, respectively, was observed in Europe from 2008 to 2017, mainly affecting older age groups [14]. In Spain, the incidence of IMD rose by 100% from 2013/14 to 2018/19 (0.42–0.84 cases per 100,000 inhabitants, respectively) coinciding with an increase of serogroup C cases in adults (25–65 years old) and serogroups W and Y in all age groups, but especially in elderly individuals. The incidence of IMD fell by 83.3% during the COVID-19 pandemic, reaching a historic nadir of 0.14 cases per 100,000 in the 2020/21 season (July 2020–June 2021) [15]. Since then, the incidence has rebounded, with 0.47 cases per 100,000 reported in 2022/23 and 0.53 cases per 100,000 described the 2023/24 [16,17].

Polysaccharide-conjugate vaccines targeting serogroups A, C, W, and Y have been developed to prevent IMD. Developing a vaccine against serogroup B has been challenging due to structural mimicry between its polysaccharide capsule and human neuronal glycoproteins, resulting in low immunogenicity [18]. Protein-based vaccines using subcapsular antigens, including the 4CMenB (Bexsero, GSK) and MenB-FHbp (Trumenba, rLP2086, Pfizer) vaccines, were developed to target serogroup B. The 4CMenB vaccine, licensed in the EEA in 2013 for use in infants over two months old, contains Neisserial heparin-binding antigen (NHBA), factor H binding protein (fHbp), *Neisseria* adhesin A (NadA), and outer membrane vesicles (OMV) from the MeNZB vaccine, predominantly with PorA encoding the variable region 2 (PorA_VR2) variant 4. The MenB-FHbp vaccine, licensed in the EEA in 2017 for use in individuals over 10 years of age, includes two lipidated recombinant fHbp from two different subfamilies [18,19]. 4CMenB was introduced in Spain in October 2015, and several regions, including Canarias, Castilla y León, Andalucía, Cataluña and Galicia, incorporated it into their immunization schedules starting in 2019 [20,21]. In January 2023, the 4CMenB vaccine was included in the National Funded Vaccination Schedule (NFVS) in Spain for infants at 2, 4, and 12 months of age [21].

Assessing vaccine efficacy for low-incidence diseases, such as IMD in clinical trials, is impractical due to the large sample size of individuals required. Instead, the licensure of MenB vaccines relies on immunogenicity results generated by the serum bactericidal antibody (SBA) assay using an exogenous source of human complement (hSBA) tested against a small number of vaccine antigen-specific indicator strains or primary strains [22,23]. The hSBA assay is limited by the number of isolates that can be evaluated, especially in studies using infant serum, as only small volumes can be collected. To address this issue, other methods to complement the hSBA assay were developed, such as the Meningococcal Antigen Typing System (MATS) and genetic MATS (gMATS), which provide large-scale, rapid, accessible, and reproducible prediction of 4CMenB protection across a broader range of strains [19]. MATS combines PorA_VR2 genotyping with an enzyme-linked immunosorbent assay to evaluate antigenic cross-reactivity and surface expression levels of fHbp, NHBA, and NadA [24]. However, due to its complexity, only a few laboratories can perform MATS. The gMATS approach evaluates strain coverage by sequencing the four antigens of the 4CMenB vaccine [25]. Additionally, the Meningococcal Deduced Vaccine Antigen Reactivity Index, which was developed independently of vaccine manufacturers, uses genomic and experimental data from published sources and is publicly available on the *Neisseria* spp. PubMLST website [26].

In Spain, very few studies have estimated 4CMenB vaccine strain coverage. Two studies, including a collection of 300 serogroup B Spanish isolates from 2008 to 2010, reported strain coverage estimates of 69% with MATS [27] and 58% with gMATS [25]. These values were lower than those in other European countries, likely due to Spain-specific genomic variations in circulating strains [28]. However, these studies were conducted before the introduction of

4CMenB in 2015, highlighting the need for updated assessments in the post-implementation period.

This study aimed to describe the genomic epidemiology of *N. meningitidis* isolates collected across 57 Clinical Microbiology Laboratories in Spain over a 12-year period (2011/12–2022/23), including isolates obtained before and after the introduction of the 4CMenB vaccine, as well as after the COVID-19 pandemic. We evaluated 4CMenB vaccine strain coverage of serogroup B isolates using genomic data and explored associations with the vaccination status of the patients. Additionally, antimicrobial susceptibility testing along with identification of antimicrobial resistance determinants was performed to evaluate susceptibility profiles.

Materials and methods

Isolates and data collection

N. meningitidis isolates obtained from blood, cerebrospinal fluid, and joint fluid in patients diagnosed with IMD were included in this study. Isolates were collected from 57 Clinical Microbiology Laboratories across 12 Spanish regions (Supplementary Figure 1) over a 12-year period from the 2011/12 to the 2022/23 seasons. Each season was defined as July of one year to June of the following year.

Patient medical records were reviewed to collect the sociodemographic characteristics including sex, age, date of IMD diagnosis, vaccination status, and any predisposing risk factors for IMD. Completion of the vaccination schedule was defined according to the manufacturer's guidelines [29].

Whole genome sequencing (WGS) and bioinformatic analysis

Short-read sequencing was performed for all isolates. DNA was extracted using the DNeasy UltraClean Microbial Kit (QIAGEN, Hilden, Germany). Libraries were prepared using the Nextera DNA Flex Kit and sequenced using the MiSeq device (Illumina, USA) according to the manufacturer's instructions. Trimmomatic (v0.39), Unicycler (v0.4.8) and SPAdes (v3.14.1) were used for raw-read trimming and *de novo* genome assembly. Assemblies were uploaded to the *Neisseria* spp. PubMLST database (https://pubmlst.org/neisseria) [30]. Genomic data were further analyzed using the "Dataset" analysis tool to determine the capsular genogroup, sequence type (ST), clonal complex (cc), *porA* and *fetA* types. The capsular serogroup was inferred from the capsular genogroup; however, we acknowledge that in rare cases, capsular expression may be absent, potentially leading to discrepancies between genogroup and serogroup. Genomes were compared with the "Genome Comparator" analysis tool using *N. meningitidis* core genome multilocus sequence typing (*N. meningitidis* cgMLST) v3.0 that includes 1329 core loci. The resulting distance matrix was evaluated with SplitsTree version 4.19.0 [31].

Genotyping of 4CMenB vaccine antigens and prediction of 4CMenB vaccine strain coverage

Genotyping of fHbp, NHBA, NadA and PorA_VR2 was conducted using the *Neisseria* spp. PubMLST database. The prediction of 4CMenB vaccine coverage for serogroup B isolates was performed using the gMATS approach. We defined a strain as gMATS "covered", "not-covered", or "unpredictable" based on the list of peptides generated by Muzzi *et al.* [25], with all NadA variants considered as not covered, as described previously.

Antimicrobial susceptibility testing

Genotyping of *gyrA* (ciprofloxacin), *penA* (β-lactams), *parC* (ciprofloxacin), and *rpoB* (rifampicin) was conducted using the *Neisseria* spp. PubMLST database. Antimicrobial susceptibility testing for penicillin G, ceftriaxone, cefotaxime, meropenem, ciprofloxacin, and rifampicin was performed using the gradient diffusion method (Etest™, bioMérieux). Isolates were incubated for 18–24 h at 37°C in a 5% CO_2 atmosphere on Mueller-Hinton agar with 5% sheep blood (BD, USA). Minimum inhibitory concentrations (MICs) were interpreted according to the EUCAST 2023 clinical breakpoints values (https://eucast.org/).

Statistical analysis

Categorical variables were compared using the chi-squared test (χ^2 test) or Fisher's exact test, as appropriate. Two-tailed p-values <0.05 were considered statistically significant.

Results

Serogroup, age, and temporal distribution

A total of 323 *N. meningitidis* isolates obtained from 322 patients with IMD were collected over 12 consecutive seasons, spanning the 2011/12 to the 2022/23 seasons. Serogroup B (MenB) accounted for 57.9% of the isolates (187/323); serogroup W (MenW) 21.4% (69/323); serogroup C (MenC) 10.4% (34/323); serogroup Y (MenY) 8.4% (27/323); and other serogroups 1.9% (6/323). Other serogroups included two isolates belonging to serogroup Z (MenZ), two isolates with a "capsule null locus" structure (cnl), one isolate from serogroup E (MenE) and one from serogroup X (MenX).

Regarding age distribution, 23.2% (75/323) of the isolates were obtained from patients over 65 years of age, 16.7% (54/323) from patients under 1 years old, and 13.9% (45/323) from patients between 1 and 4 years old (Figure 1A). MenB predominated among patients less than 1 year of age (81%; 44/54), 1–4 years (84%; 38/45), 5–9 years (79%; 23/29), and 10–14 years (62%; 8/13). In contrast, MenC was more prevalent among patients 25–44 years old (44.8%; 13/29). MenW and MenY were identified across all age groups but predominated among the oldest age groups. Among patients 45–64 years of age and those over 65 years, MenW accounted for 31.6% (12/38) and 38.7% (29/75), respectively, while MenY accounted for 10.5% (4/38) and 14.7% (11/75), respectively.

MenB predominated throughout most seasons of the study, except during the 2018/19 and 2019/20 seasons (Figure 1B). Regarding MenW and MenY, both serogroups were present in only small numbers during the first five seasons. However, a notable increase in MenW was observed in the 2016/17 season, peaking during the 2018/19 and 2019/20 seasons. A concurrent peak in MenY cases was also recorded during the same seasons. Following 2019/20, an overall decrease in cases was observed, likely attributable to public health measures implemented during the COVID-19 pandemic that reduced the transmission of *N. meningitidis*. In the seasons following the COVID-19 pandemic, only MenB, MenW, and MenY isolates were identified.

Population structure and genetic diversity

A total of 106 different STs were identified among the 323 isolates included in this study, which were distributed across 22 cc. The most prevalent cc was cc11 (28.5%; 92/323), followed by cc213 (23.2%; 75/323), cc269 (7.7%; 25/323), cc32 (6.8%; 22/323) and cc461 (6.5%; 21/323). Eleven isolates (3.4%), distributed among eight STs, were not assigned to any cc (Figure 2A). Interestingly, four isolates belonging to ST-10603, ST-15630, ST-15962, and ST-17035, were not assigned to any cc by the PubMLST database. However, after cgMLST analysis, these isolates clustered with cc269 isolates (Figure 2B). Therefore, we assumed that they were closely related to this cc and conducted all subsequent analysis accordingly (Supplementary Table 1).

A broad genetic diversity was observed among the 187 MenB isolates, with 71 STs that clustered into 13 different cc identified. The most prevalent were cc213 (40.1%; 75/187), followed by cc269 (12.8%; 24/187), cc32 (11.2%; 21/187), cc461 (11.2%; 21/187), and to a lesser extent, cc41/44 (5.4%; 10/187), and cc162 (4.3%; 8/187). The predominant subtypes were P1.22,14 (40.1%; 75/187) and P1.22,9 (11.8%; 22/187), highly related to cc213 and cc269, respectively. Interestingly, no changes were observed in the circulating cc before and after the COVID-19 pandemic, with cc213 remaining the most prevalent (Supplementary Figure 2).

Regarding the MenC isolates, most belonged to cc11 (82.4%; 28/34) and subtype P1.5-1,10-8 (50%; 17/34), or to a lesser extent, P1.5,2 (26.5%; 9/34). The remaining six each belonged to a distinct cc, including cc35, cc103, cc162, cc174, cc1572 and one ST-11429 isolate not assigned to any cc. The majority of MenW isolates belonged to cc11 (89.9%; 62/69) and subtype P1.5,2 (91.3%; 63/69), demonstrating a high degree of clonality within MenW. The remaining seven belonged to cc22 (7.3%; 5/69), one cc865, and

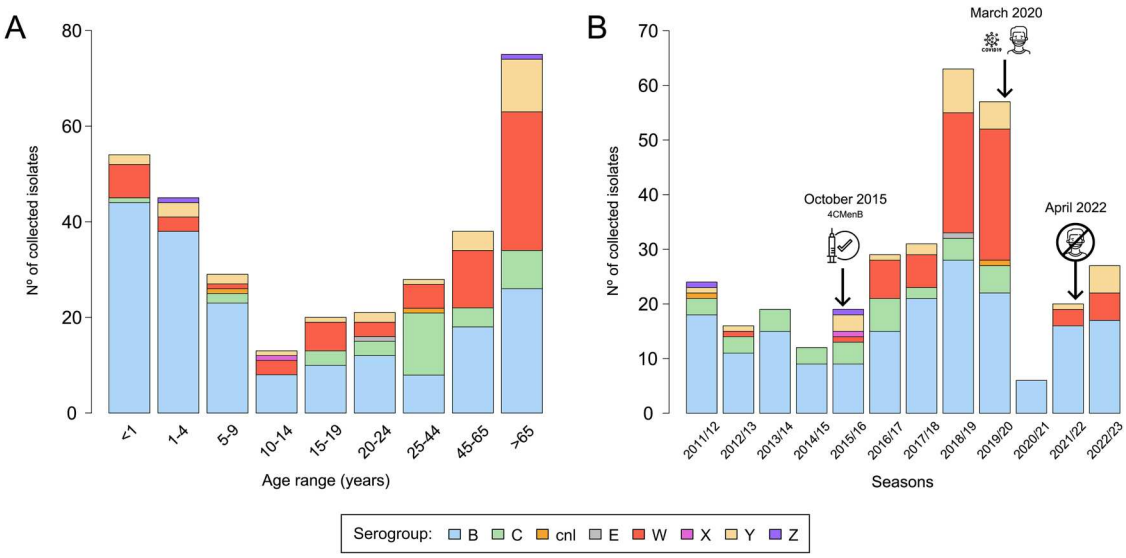

Figure 1. Distribution of 323 invasive *N. meningitidis* isolates collected in this study by (A) age and (B) season. Colors indicate the capsular serogroup (cnl: capsule null locus). Black arrows indicate the month and year when the 4CMenB vaccine was introduced in Spain, as well as the implementation and subsequent lifting of COVID-19 social restrictions in the country.

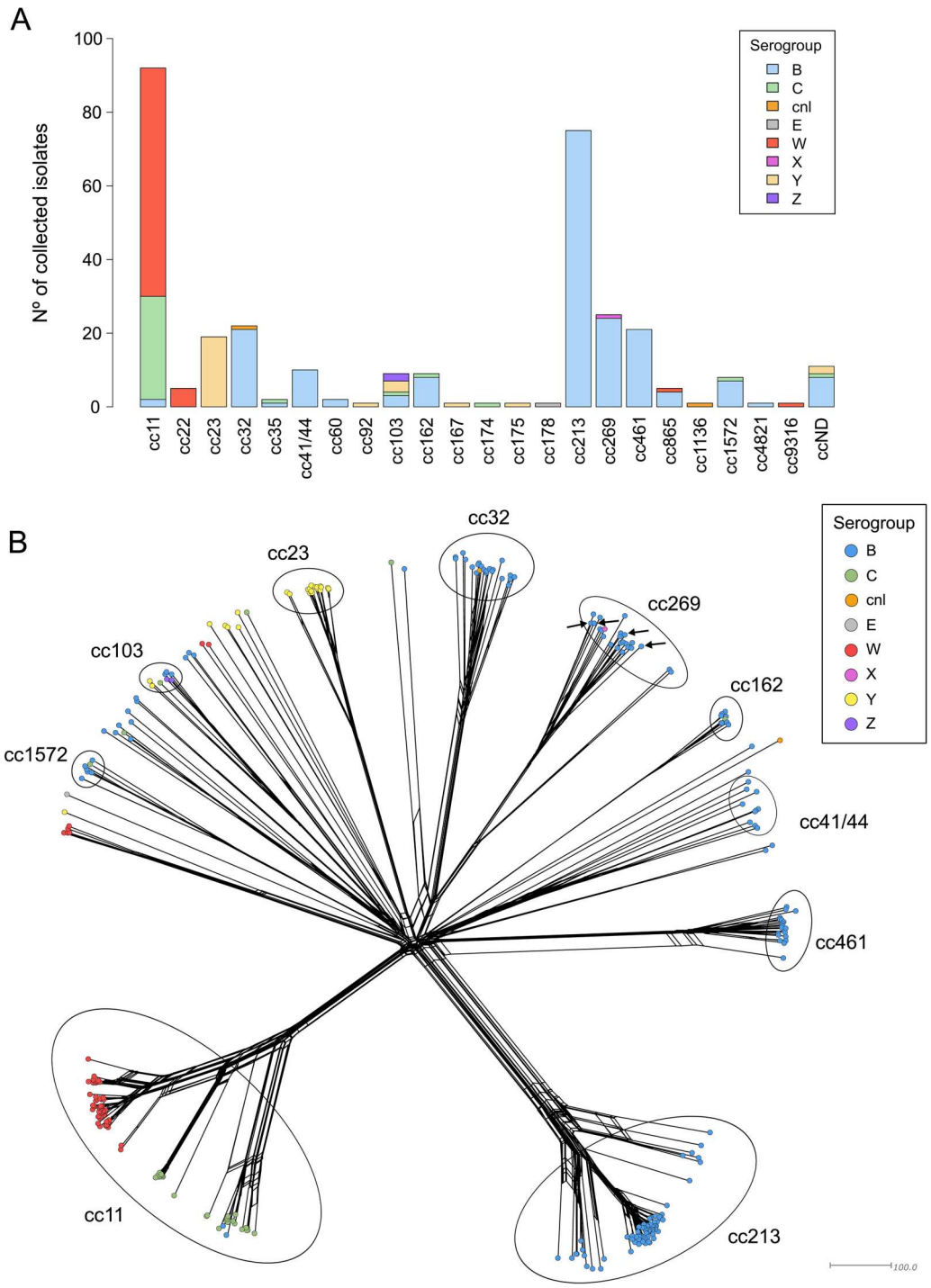

Figure 2. Population description of 323 invasive *Neisseria meningitidis* isolates collected in this study. (A) Distribution of serogroups per clonal complex (ccND: clonal complex not defined; cnl: capsule null locus), colors indicate capsular serogroup. (B) Genomes were compared using the *N. meningitidis* cgMLST v3.0 scheme. The resulting distance matrices were assessed with SplitsTree4 version 4.19.0 using the NeighborNet algorithm to construct the tree. Each dot represents one genome, the color of the dot indicates the capsular serogroup of the isolate. The 10 major clonal complexes are highlighted with a black circle. The black arrows indicate the four isolates closely related to cc269 that were not assigned to that clonal complex.

one cc9316. Within MenY, cc23 predominated (70.4%; 19/27) and the subtypes identified were P1.5-1,10-1 (33.3%; 9/27), P1.5-2,10-1 (25.9%; 7/27), and P1.5-2,10-28 (11.1%; 3/27), all of which were highly related to cc23. The remaining eight belonged to cc103 (3/27), one cc92, one cc167, and one cc175, and two isolates (ST-1768 and ST-5436) not assigned to any cc.

Prediction of 4CMenB vaccine strain coverage of serogroup B isolates by gMATS

Prediction of the coverage of the 4CMenB vaccine against the 187 MenB isolates included in the study was assessed using gMATS. This method determined that 28.9% of the isolates were predicted to be covered by 4CMenB, while 42.8% were predicted to not be covered, and the coverage was unpredictable in the

Table 1. Combinations of antigens for 4CMenB vaccine strain coverage in serogroup B isolates as determined by gMATS.

	n	%
3 antigens		
fHbp+NHBA+PorA_VR2	0	N/A
2 antigens		
fHbp+NHBA	8	14.8%
fHbp+PorA_VR2	1	1.8%
NHBA+PorA_VR2	3	5.6%
1 antigen		
fHbp	19	35.2%
NHBA	23	42.6%
PorA_VR2	0	N/A

remaining 28.3%. No significant differences were observed in the proportion of covered or not covered isolates across the seasons of the study period (Supplementary Figure 3).

A total of 34 (18.2%) isolates showed NHBA peptides defined as covered by gMATS, 28 (15%) isolates exhibited fHbp peptides defined as covered, and only 4 (2.1%) exhibited PorA_VR2-4. The most common combination was NHBA+fHbp, with no isolate covered by all three antigens simultaneously (Table 1).

The predicted coverage by gMATS among the different cc varied greatly (Figure 3). The cc with the highest proportion of covered isolates were cc162, cc32 and cc41/44, with 100%, 95.2%, and 70% of isolates covered, respectively. The lowest proportion of covered isolates was observed among cc213 and cc269, with 5.3% and 20.8% of isolates being covered, respectively. Conversely, gMATS determined that 84% of cc213 isolates and 50% of cc269 isolates were not covered by 4CMenB. Interestingly, vaccine coverage was unpredictable for all the cc461 isolates.

Characteristics of 4CMenB antigens among serogroup B isolates

Antigenic studies revealed 68 distinct fHbp peptides, of which 47 were identified in single isolates, and 20 were identified as new peptides not previously described (Figure 4A). Variant 3 peptides were the most prevalent among MenB isolates (92/187; 49.2%), followed by variant 1 peptides (61/187, 32.6%) and variant 2 peptides (34/187, 18.2%). The most frequent peptide was fHbp-3.45 (37/187; 19.8%), primarily associated with cc213 and classified as not covered by gMATS. This was followed by fHbp-1.1 (15/187; 8%), which is the peptide included in the 4CMenB vaccine, and predominantly linked to cc32.

For NHBA, 28 distinct peptides were identified, with 12 occurring in single isolates, and 4 were identified as new peptides not previously described (Figure 4B). The most common peptide was NHBA-18 (69/187; 36.9%), strongly associated with cc213 and classified as not covered, followed by NHBA-118 (21/187; 11.2%), an unpredictable peptide linked to cc461. Peptide NHBA-2, included in the 4CMenB vaccine, was detected in only five isolates (2.7%) from cc41/44.

Regarding PorA_VR2, 36 variants were identified, with 22 found in single isolates, and 1 was identified as a new variant not previously described (Figure 4C). The most frequent variants were PorA_VR2-14 (84/187; 44.9%) and PorA_VR2-9 (23/187; 12.3%), both classified as not covered, and predominantly associated with cc213 and cc269, respectively. PorA_VR2-4, included in the 4CMenB vaccine, was identified in only four isolates (2.1%), one belonging to cc41/44 and three to cc162.

4CMenB vaccination status and clonal complex patterns in individuals with serogroup B IMD

Information on 4CMenB vaccination status was available for 82.4% (154/187) of patients with MenB IMD. Among these, 15.6% (24/154) had received at least

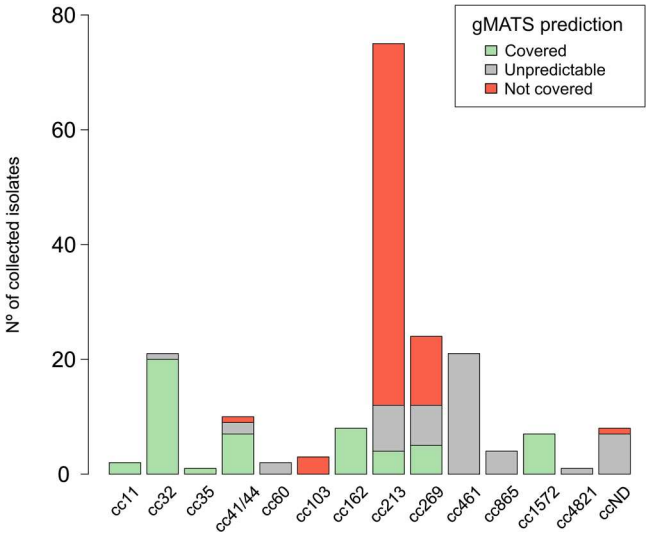

Figure 3. Prediction of 4CMenB vaccine strain coverage with the gMATS approach of serogroup B isolates per clonal complex (ccND: clonal complex not defined).

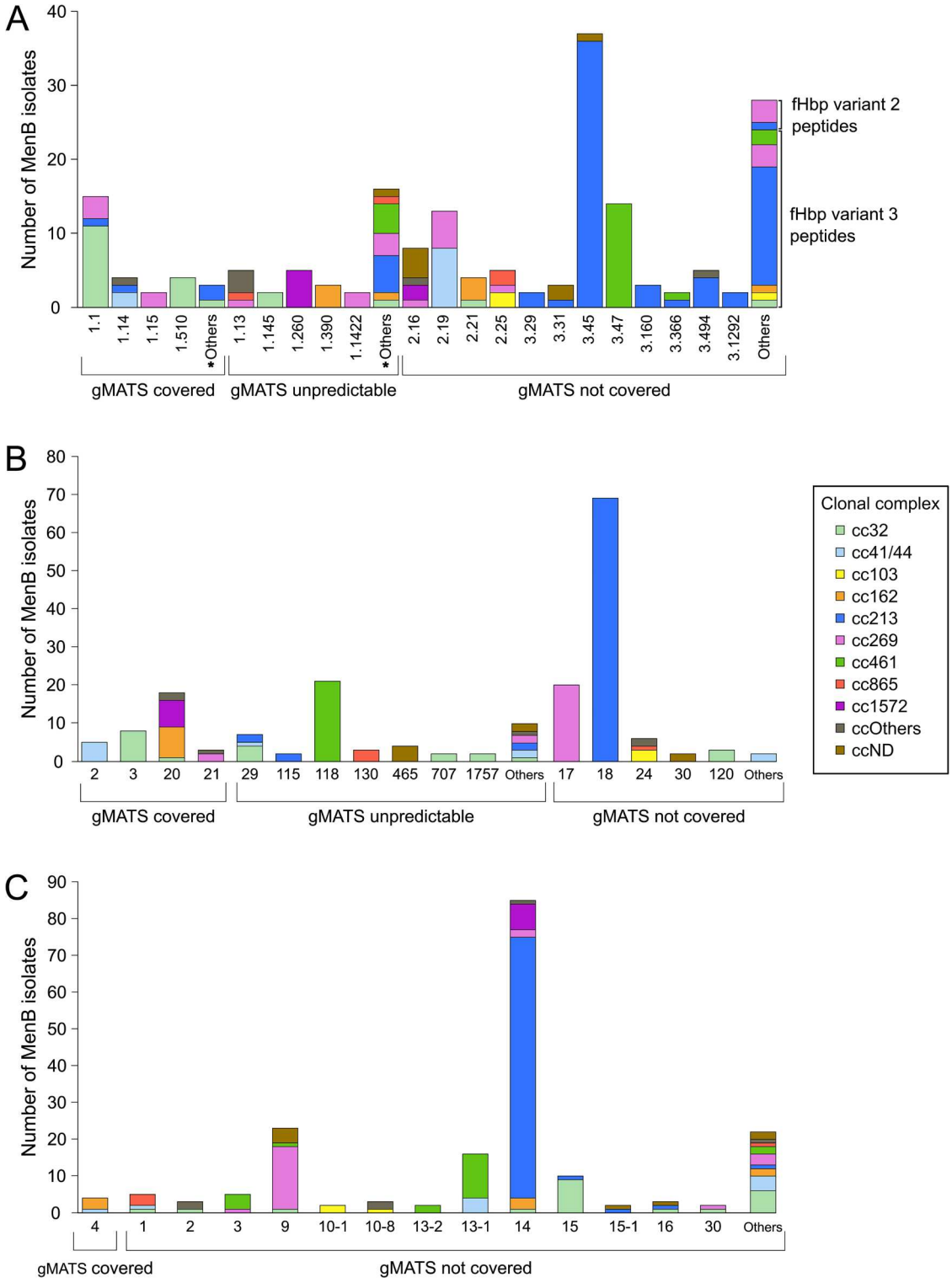

Figure 4. Bar chart representation of (A) fHbp peptides, (B) NHBA peptides, and (C) PorA_VR2 variants identified among serogroup B isolates. Peptides are grouped based on their classification as covered, unpredictable or not covered by the gMATS approach. Colors indicate the clonal complexes of the isolates carrying each peptide (ccOthers: clonal complexes identified only once or twice in this study; ccND: clonal complex not defined). 4CMenB antigen peptides identified only once in this study are grouped under category "Others", while category "Others*" refers specifically to variant 1 fHbp peptides.

one dose of 4CMenB, but only 50% (12/24) had completed the full vaccination schedule (Table 2). Regarding the 4CMenB strain coverage of the MenB isolates causing IMD in fully or partially vaccinated patients, 12.5% (3/24) were predicted to be covered by gMATS, including two cc32 and one cc1572 isolate, 66.7% (16/24) were predicted to not be covered, all belonging to cc213, and the remaining 20.8% (5/24) were unpredictable, comprising two cc461 isolates, one cc60 isolate, one cc213 isolate, and one ST-11429 isolate with no assigned cc. A similar proportion of cc213 cases was observed in fully

Table 2. Demographic and vaccination history of 4CMenB-vaccinated patients with serogroup B IMD episodes, including genomic and prediction of 4CMenB vaccine strain coverage for their corresponding isolates.

ID	Sex/ Age	Vaccination schedule	4CMenB doses received	Date last 4CMenB dose received	Diagnosis date	Serogroup/Clonal complex	gMATS prediction
V1	F/9	Complete	2	2015 Apr	2016 Jan	B:ccND	Unpredictable
V2	M/2	Complete	2	2016 Jun	2016 Aug	B:cc213	Not covered
V3	M/12	Complete	2	2016 Sep	2017 Feb	B:cc213	Not covered
V4	M/4	Complete	2	2017 May	2018 Feb	B:cc213	Not covered
V5	M/8m	Complete	2	2018 Oct	2019 Jan	B:cc213	Not covered
V6	F/4	Complete	2	2016 Dec	2019 Feb	B:cc213	Not covered
V7	M/3	Complete	4	UNK	2019 Apr	B:cc213	Not covered
V8	M/4	Complete	2	2017 Sep	2019 May	B:cc461	Unpredictable
V9	M/3	Complete	4	2017 May	2019 Dec	B:cc213	Not covered
V10	F/4	Complete	3	2019 Oct	2021 Nov	B:cc213	Not covered
V11	M/9m	Complete	2	2021 Dec	2022 May	B:cc213	Not covered
V12	F/20	Complete	2	2021 Oct	2023 May	B:cc1572	Covered
V13	M/7m	Uncomplete	1	2016 Nov	2016 Dec	B:cc213	Not covered
V14	F/8m	Uncomplete	1	UNK	2018 May	B:cc213	Unpredictable
V15	M/1	Uncomplete	1	2017 Apr	2018 Jul	B:cc213	Not covered
V16	F/2	Uncomplete	2	2018 Feb	2019 Jul	B:cc213	Not covered
V17	M/4m	Uncomplete	1	2019 Oct	2019 Nov	B:cc461	Unpredictable
V18	M/4	Uncomplete	1	2019 May	2019 Nov	B:cc213	Not covered
V19	M/5	Uncomplete	2	2016 Oct	2020 May	B:cc213	Not covered
V20	F/6m	Uncomplete	1	2021 Nov	2022 Jan	B:cc32	Covered
V21	M/3	Uncomplete	2	2019 Sep	2022 Apr	B:cc60	Unpredictable
V22	F/3m	Uncomplete	1	2022 Sep	2022 Dec	B:cc213	Not covered
V23	F/5m	Uncomplete	1	2022 Dec	2023 Jan	B:cc32	Covered
V24	M/1	Uncomplete	2	2022 Mar	2023 Feb	B:cc213	Not covered

UNK: unknown; ccND: clonal complex not defined.

versus partially vaccinated patients (75% vs 66.7%, respectively: $p = 0.99$, RR: 1.13, 95% CI: 0.64-2.03). However, a significantly higher proportion of cc213 cases was observed in vaccinated compared to non-vaccinated patients (70.8% vs 35.4%; $p = 0.003$, RR: 2, 95% CI: 1.35-2.76). No other significant trends in cc distribution relative to vaccination status were identified (Table 3).

A single IMD case caused by a predicted covered isolate was identified in a fully vaccinated patient (V12) with complement deficiency. This patient had experienced a first IMD episode in 2007 during infancy (isolate not available). A second episode occurred in July 2021, caused by a MenB:cc41/44 strain, while unvaccinated. Despite receiving two doses of 4CMenB, a third episode caused by a covered MenB:cc1572 strain was presented. Additionally, two IMD cases involving predicted covered isolates occurred in partially vaccinated individuals (V20 and V23). Both patients were under one year of age, had received a single dose of 4CMenB before IMD, and had no relevant medical history.

Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed in 304 isolates (94.1%) showing that 13.5% (41/304) of the isolates exhibited resistance to penicillin G (PEN^R), 0.3% (1/304) to ciprofloxacin (CIP^R), 0.3% (1/304) to rifampicin (RIF^R), and no isolates showed resistance to cefotaxime, ceftriaxone, or meropenem.

Table 3. Clonal complex distribution of serogroup B isolates versus vaccination status of patients at the time of the IMD episode.

	Complete vaccination status (n = 12)		Incomplete vaccination status (n = 12)		Vaccinated[a] (n = 24)		Non-vaccinated (n = 130)		All (n = 154)		RR vaccinated versus non-vaccinated (95% CI)		P value[b]
Clonal complex	n	%	n	%	n	%	n	%	n	%			
CC103							3	2.3	3	1.9	n/a		
CC11							2	1.5	2	1.3	n/a		
CC1572	1	8.3			1	4.2	6	4.6	7	4.5	0.90	(0.14–5.21)	0.999
CC162							7	5.4	7	4.5	n/a		
CC213	9	75	8	66.7	17	70.8	46	35.4	63	40.9	2	(1.35–2.76)	**0.003**
CC269							19	14.6	19	12.3	n/a		
CC32			2	16.7	2	8.3	19	14.6	21	13.6	0.57	(0.15–1.92)	0.532
CC41/44							9	6.9	9	5.8	n/a		
CC461	1	8.3	1	8.3	2	8.3	11	8.5	13	8.4	0.98	(0.25–3.52)	0.999
CC4821							1	0.8	1	0.6	n/a		
CC60			1	8.3	1	4.2			1	0.6	n/a		
CC865							3	2.3	3	1.9	n/a		
ccND	1	8.3			1	4.2	4	3.1	5	3.2	1.35	(0.21–8.35)	0.577

RR: Risk Ratio; Blank boxes correspond to 0; ccND: clonal complex not defined.
[a]Vaccinated group includes those with complete and incomplete vaccination status.
[b]Bold type = significant (<0.05)

No association was observed between antimicrobial susceptibility profiles and serogroups (Supplementary Table 2).

In relation to PEN^R, 82.9% (34/40) of resistant isolates displayed various *penA* alleles that simultaneously encoded the five characteristic substitutions F504L, A510V, I515V, H541N, and I566V in PBP2, associated with resistance in *N. meningitidis* [32]. The remaining PEN^R isolates harbored either the *penA*327 allele with only the first four substitutions (7.3%; 3/41) or expressed *penA* alleles without any mutations (9.8%; 4/41), such as the wild-type alleles *penA*1 and *penA*22 [33]. In general, isolates containing mutations in PBP2 presented a higher MIC than those with wild-type alleles (Figure 5). No β-lactamase encoding gene was detected in any isolate of this study.

Although no resistance to 3GC was observed, five isolates exhibited a cefotaxime MIC near the clinical breakpoint (two isolates with MIC = 0.125 µg/mL and three with MIC = 0.094 µg/mL). All five belonged to MenC:cc11, one carried the *penA*1 allele, while the remaining four carried the *penA*327 allele, which includes the G545S substitution in PBP2.

The RIF^R isolate (MIC = 0.5 µg/mL) belonged to MenW:cc22 and exhibited the *rpoB*4 allele. No previously reported mutations associated with rifampicin resistance in the *rpoB* gene were detected [34,35]. Additional analysis of the *rpoB* gene did not reveal any novel mutation linked to rifampicin resistance.

The CIP^R isolate (MIC = 0.023 µg/mL) belonged to MenB:cc213 and carried the *gyrA*403 allele, a newly described allele in the PubMLST database, which encodes an A92P substitution not previously reported. Another MenB:cc213 isolate in this study carried the same allele and presented a MIC close to the clinical breakpoint value (MIC = 0.016 µg/mL). No previously reported mutations associated with ciprofloxacin resistance in the *gyrA* or *parC* were detected [9,11,12,36,37].

Discussion

This study provides an in-depth molecular characterization of genetic diversity and predicted 4CMenB vaccine coverage in a collection of 323 *N. meningitidis* isolates responsible for IMD in Spain during the 2011/12 and 2022/23 seasons. By integrating WGS with antimicrobial susceptibility testing, the study also assessed the relationship between antimicrobial resistance markers and the susceptibility profiles of the isolates.

MenB was identified as the predominant serogroup (57.9%) in our study, with a higher prevalence among paediatric and young adult populations up to the age of 25, followed by MenW (21.4%), MenC (10.4%), and MenY (8.4%). These results are consistent with previous European surveillance reports [1,2]. Beginning in the 2016/17 season, an increase in MenW and MenY cases was observed in Spain, with the largest proportion of cases associated with older adults. Since 2009, the emergence of MenW cases associated with cc11 in England and MenY cases in Northern European countries (Finland, Norway, and Sweden) has been reported [38,39], and our results are consistent with these findings. To address this situation, the MenACWY vaccine was implemented in the United Kingdom and the Netherlands, where it successfully controlled the spread of MenW [40]. Similarly, Spain incorporated the MenACWY vaccine into its NFVS

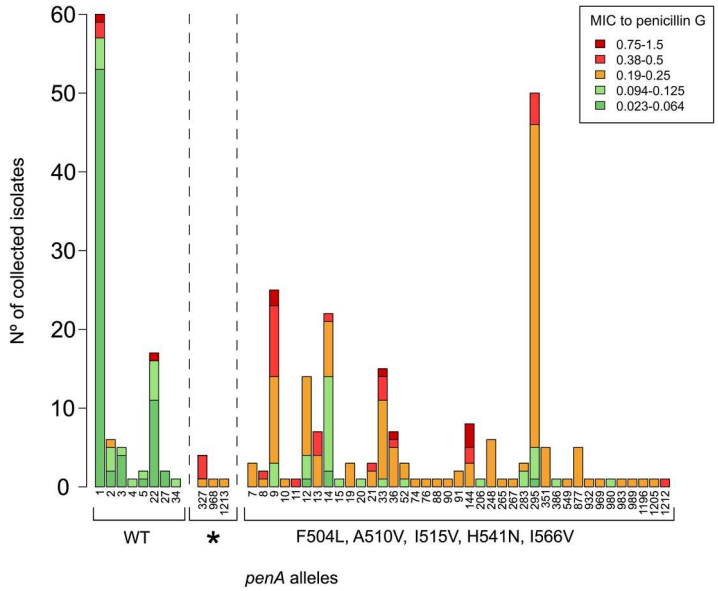

Figure 5. Bar chart representation of the *penA* alleles identified among 304 *N. meningitidis* isolates, along with the corresponding MIC values. The alleles are grouped based on the number of PBP2 mutations encoded, and the color represents the MIC of isolates carrying each *penA* allele. An asterisk indicates alleles encoding the following PBP2 mutations: F504L, A510V, I515V, and H541N.

in 2020, replacing the monovalent MenC vaccine for 12 year olds [20]. This highlights the importance of adapting vaccination programs in response to the evolving epidemiology of IMD.

As has been demonstrated in other infections caused by airborne-transmitted pathogens, including *Streptococcus pneumoniae* and *Haemophilus influenzae* [41], cases of IMD significantly decreased after the 2019/20 season, primarily due to the social restrictions imposed during the COVID-19 pandemic. Following the relaxation of these restrictions the present study observed a subsequent rise in IMD cases, with MenB re-emerging as the most prevalent serogroup, circulating close to pre-pandemic levels and accounting for 70.2% of all isolates collected between the 2021/22 and 2022/23 seasons. This trend was also observed across the EEA, where MenB accounted for 62% of IMD cases in 2022 [1]. Interestingly, no changes were detected in the circulating cc before and after the pandemic. As for MenW and MenY, both serogroups re-emerged during the last two seasons of the study, accounting for 17% and 12.8%, respectively, although neither has reached pre-pandemic levels [17]. A rebound of MenY cases was reported across the EEA in 2022, making it the second most common serogroup, responsible for 16% of IMD cases that year [1]. However, no such rebound was observed in our study. This could have been influenced by the introduction of the MenACWY vaccine into the Spanish NFVS in 2020, as well as the reduced transmission of *N. meningitidis* during the pandemic. Nevertheless, continued surveillance in upcoming seasons will be necessary to identify any potential "delayed" resurgence of these serogroups, as has occurred in the past. In the United States, MenY also experienced an abrupt resurgence, representing 35% of IMD cases in 2023, mainly associated with the ST-1466 and cc174 [42]. However, in the present study, MenY cases were mainly associated with cc23, consistent with previous data from France [43]. Finally, while MenC cases remained stable during the study period prior to the pandemic, no MenC isolates have been identified among our isolates since the 2019/20 season. These findings underscore the rapidly evolving epidemiology of IMD and highlight the critical importance of continued surveillance to assess the effectiveness of control measures and guide future public health strategies.

The genomic analysis of the isolates revealed greater genetic diversity among MenB compared to other serogroups. The main cc identified among MenB isolates in this study were cc213 (40.1%) followed by cc269 (12.8%). Similar to our results, previous findings noted that some STs related to cc269 do not meet the MLST-based definition for assignment to this complex [44]. As a result, such STs may often be overlooked in MLST-based studies. Genomic approaches, such as cgMLST, provide a more comprehensive view of strain relationships. Indeed, a broader lineage (Lineage 2) was described based on whole-genome sequencing data, which includes cc269 and its related unassigned STs [45]. Our findings further support the inclusion of these STs within the cc269-related lineage. Notably, previous studies have documented the increasing prevalence of cc213 in Spain, where it accounted for 17.7% of MenB isolates in 2009–2010 [28] rising to 32.8% in 2015–2018 [46]. This trend has not been observed in neighboring countries, where cc41/44 and/or cc32 were the most detected cc. Thus, in a study conducted in Portugal with isolates collected from 2012 to 2020, cc41/44 was the most frequently detected (26.3%), followed by cc213 (16.3%) [6]. In contrast, cc32 (32%) prevailed in France in 2018-2019, followed by cc41/44 (13.3%) and cc213 (11.9%) [47]. Similar trends were observed in other European countries, such as England, Switzerland, and the Netherlands [48–50]. Notably, in our study, cc32 and cc41/44 were a minority among MenB isolates (11.2% and 5.4%, respectively). While none of these countries reported a prevalence of cc213 as high as that observed in the present study, the prevalence rates of this cc are, nonetheless, notable and have been increasing over time. These results highlight the unique genomic epidemiology of the MenB isolates responsible for IMD in Spain, characterized by the exceptional prevalence of cc213.

Regarding the study of vaccine coverage using the gMATS approach, our study revealed that only 28.9% of the MenB isolates were covered by the 4CMenB vaccine. This coverage increased to 43% using the estimate defined by Muzzi *et al.*, as it considers the proportion of covered strains plus half the proportion of unpredictable strains. This estimate is considerably lower in comparison to the 58% identified for MenB isolates collected in Spain between 2009 and 2010 [25]. Coverage estimates from strains collected during similar periods in other European countries are usually higher: 84% in Finland (2010/11–2016/17) [51], 70.7% in France (2018–2019) [47], 86.6% in Poland (2010–2016) [52], and 73% in the Netherlands (2017–2019) [48]. Several factors may explain these geographical variations. Firstly, the prevalence of fHbp variant 3 peptides, which are predicted as not being covered by gMATS, has increased over the past decade in association with the rise of isolates belonging to cc213. In our study, 49.2% of MenB isolates carried variant 3 peptides, compared to only 21.7% of Spanish MenB isolates from 2009 to 2010 and 36.6% of Spanish MenB isolates from 2015 to 2018 that carried this variant [28,46]. In contrast, other European countries report an even lower prevalence of variant 3 peptides. For instance, only 29.6% of MenB isolates in England from 2014/15 to 2017/18 harbored variant 2 or 3 peptides [50], compared to

26% of Finnish MenB isolates from 2010/11 to 2016/17 [51] and 36.3% of French MenB isolates during 2018–2019 [47]. Additionally, fHbp-1.1 (included in the 4CMenB vaccine) was found in only 8% of our MenB isolates, whereas it was identified in 19.33% of Spanish MenB isolates from 2009–2010 [28]. Secondly, NHBA-2 and PorA_VR2-4 variants (both included in the 4CMenB vaccine) were present in only 2.7% and 2.1% of our MenB isolates, respectively. Classically, these have been associated with cc41/44 [47], which as mentioned before, was rarely identified among MenB isolates in the present study (5.4%). Thirdly, the most prevalent variants for each antigen in our MenB isolates were fHbp-3.45 (19.8%), NHBA-18 (36.9%), and PorA_VR2-14 (44.9%). All three classified as not covered by gMATS and strongly associated with cc213, which, as demonstrated in this study, remains the principal cc in Spain. Additionally, the fact that the proportion of MenB cases caused by cc213 is twice as high in vaccinated patients compared to non-vaccinated patients (70.8% vs 35.4%; $p = 0.003$) highlights the growing threat posed by the expansion of cc213 to both vaccinated and unvaccinated individuals. Similar to our study, proportionally more cases in vaccinees were associated with cc213 strains compared to non-vaccinees (22.9% vs 9.6%; $p < 0.01$) in England [50]. This underscores the critical need for continued monitoring of vaccine coverage at both genomic and phenotypic levels, particularly given the inclusion of the 4CMenB vaccine in the Spanish NFVS for infants since 2023. Additionally, although the MenB-FHbp vaccine is not currently in use for children under 10 years old, further studies evaluating its strain coverage would be valuable.

Regarding the antimicrobial susceptibility, all the isolates tested were susceptible to ceftriaxone and cefotaxime, the first-line empirical treatments for IMD, as has been commonly reported by other European countries [53]. However, five isolates exhibited cefotaxime MIC values close to the clinical breakpoint; with four harboring the *penA*327 allele, encoding the G545S substitution in PBP2, a mutation previously associated with increased MIC values to cefotaxime in *N. meningitidis* [7,54–56]. Additionally, 13.5% of the isolates were PEN^R, a rate comparable to Portugal (15.9% from 2012 to 2020) but higher than the United Kingdom (2.7% from 2010/11 to 2018/19) [6,8]. Genetic analysis revealed that PEN^R was primarily conferred by mutations in the *penA* gene, with no evidence of β-lactamase production. Nevertheless, some resistant strains lacked known mutations in PBP2, suggesting alternative mechanisms or novel mutations contributing to resistance. RIF^R is rare and typically linked to mutations in the *rpoB* gene encoding the β subunit of RNA polymerase [7,57,58]. The only RIF^R isolate identified in this study exhibited low-level resistance without detectable *rpoB* mutations. This finding implies that resistance may result from other mechanisms, such as alterations in membrane permeability or efflux pump activity [59]. Regarding CIP^R, a novel *gyrA*403 allele encoding an A92P substitution was identified in two MenB: cc213 isolates (MIC = 0.023 and 0.016 μg/mL). This mutation has previously been reported in quinolone-resistant *N. gonorrhoeae* but not in *N. meningitidis* [60,61]. Circulating *N. meningitidis* strains causing IMD in Spain remain largely susceptible to standard treatments, but emerging resistance to rifampicin, ciprofloxacin, and cefotaxime [8,54] underscores the need for ongoing antimicrobial susceptibility surveillance at national and international levels to ensure treatment efficacy and maintain effective IMD control.

The limitations of this study include potential sampling bias, as only recovered strains were studied, which may not represent all IMD cases across Spain. National guidelines recommend obtaining a sample for microscopy, culturing and PCR [5,62]. However, not all strains causing IMD can always be recovered. Our collection of *N. meningitis* causing IMD in Spain represent 10.8% of all IMD cases confirmed in the country, spanning the same time periods. Additionally, isolates were voluntarily contributed by microbiology laboratories across Spain, and due to geographical proximity, a higher number of isolates in this study were collected in Catalonia, which may be more represented than other regions. IMD epidemiology may vary among regions in Spain; for instance, Melilla reported no MenACWY cases in 2023, possibly due to its early adoption of MenACWY vaccination for adolescents in 2017, while other regions introduced it later, from 2019 to 2020. Despite these biases, our collection seems to be a true representation of the Spanish epidemiology, as we have been able to detect minority serogroups and clonal complexes among our collection, and, moreover, the most prevalent serogroups and clonal complexes were detected in different regions of Spain in different seasons. Our study is also limited by the use of genotyping-based approaches. gMATS is considered a conservative method, as it does not account for the contribution of the NadA antigen, minor OMV constituents, low-frequency or novel antigen variants, and cooperative effects among antigens, as well as the *in vivo* role of the human complement system. As a result, the actual vaccine coverage of our MenB panel may differ from the predictions provided in this study. To overcome this limitation, further research employing phenotypic methodologies, such as MATS and hSBA, is necessary to accurately evaluate vaccine strain coverage, particularly since 28.3% of MenB isolates remain unpredictable by gMATS, with a significant proportion belonging to cc461 (39.6%).

Conclusions

In summary, a high prevalence of MenB isolates (57.9%) was registered among *N. meningitidis* isolates causing IMD in Spain, especially after the COVID-19 pandemic (70.2%). A high diversity of cc was found among MenB isolates, with cc213 being the most prevalent (40.1%) and presenting the highest rate of isolates not covered (84%) by gMATS. This cc was also found to be significantly more prevalent among the 4CMenB-vaccinated individuals presenting IMD than among the non-vaccinated individuals, which may pose a growing threat for IMD prevention. Regarding antimicrobial susceptibility, no resistance to 3GC was detected, and resistance to rifampicin and ciprofloxacin was rare. Further studies addressing epidemiological surveillance, vaccine reactivity prediction, and antimicrobial susceptibility testing are essential for assessing potential health threats and ensuring effective prevention, prophylaxis, and treatment of IMD.

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Author contribution

Isolates and clinical data were collected by CMA, ML, ABB, JC, EC, MAGL, AMN, DNC, MAO, BP, APA, MDQ, AR, AR, ERG, CS, AS, BV and NL. Whole genome sequencing and antimicrobial susceptibility testing was performed by JRG and AMC. Data and genomic analysis by JRG, AMC, AMM and GP. The manuscript was written by JRG and JJGL, and supported, commented and edited by all the other co-authors.

Ethical approval

The study was approved by the Ethics Committee of Vall d'Hebron Hospital, reference number PR(AG)17/2022. The study was conducted in accordance with the principles laid out in the Declaration of Helsinki and in accordance with the principles of Good Clinical Practice.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The genome assembly sequences used in this study were deposited in the PubMLST *Neisseria* spp. database. The identification numbers for all isolates are provided in Supplementary Table 1.

ORCID

Josep Roca-Grande http://orcid.org/0000-0002-7177-1684
Alba Mir-Cros http://orcid.org/0000-0003-4827-9824
Carmen Muñoz-Almagro http://orcid.org/0000-0001-5586-404X
Mayli Lung http://orcid.org/0000-0002-2692-4370
Alba Bellés-Bellés http://orcid.org/0000-0001-6277-1239
Jordi Càmara http://orcid.org/0000-0002-0817-0931
Emilia Cercenado http://orcid.org/0000-0002-5279-3773
Andrea Martín-Nalda http://orcid.org/0000-0002-1715-153X
Albert Moreno-Mingorance http://orcid.org/0000-0002-3073-5046
Daniel Navarro de la Cruz http://orcid.org/0000-0002-7556-5861
Guillem Puigsech-Boixeda http://orcid.org/0009-0001-6036-5429
M. Dolores Quesada http://orcid.org/0000-0001-8360-3787
Alba Rivera http://orcid.org/0000-0002-9709-6162
Enrique Ruiz de Gopegui http://orcid.org/0000-0002-8752-6894
Aleix Soler-Garcia http://orcid.org/0000-0001-7190-0653
Belén Viñado http://orcid.org/0000-0001-6212-5886
Nieves Larrosa http://orcid.org/0000-0001-8808-0233
Juan José González-López http://orcid.org/0000-0003-2419-5909

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