Molecular characterization of a collection of *Neisseria meningitidis* isolates from Croatia, June 2009 to January 2014

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In the last decade, the incidence of invasive meningococcal disease (IMD) in Croatia remained stable at approximately 1 case per 100 000 inhabitants, affecting mainly children aged ≤5 years. We report the molecular characterization of meningococci causing IMD occurring from June 2009 to January 2014 in Croatia. Genomic DNA from 50 clinical isolates was analysed for serogroup, multilocus sequence typing and allele type of the two outer membrane protein genes, *porA* and the iron-regulated *fetA*. Furthermore, 22 of them were characterized by using whole-genome sequencing to define the meningococcal vaccine four-component meningococcal serogroup B (4CMenB) antigen genes factor H-binding protein (*fHbp*), *Neisseria* heparin-binding antigen (*nhba*) and *Neisseria* adhesin A (*nadA*) and the antimicrobial target resistance genes for penicillin (penicillin binding protein 2, *penA*), ciprofloxacin (*DNA gyrase subunit A, gyrA*) and rifampicin (*β*-subunit of RNA polymerase, *rpoB*). The Etest was used to phenotypically determine the antimicrobial susceptibility of isolated meningococci. The main serogroup/clonal complex combinations were MenB cc41/44, MenC/cc11, MenW/cc174 and MenY/cc23. PorA P1.7-2, FetA F5-5 and F1-5 were the most represented through the serogroups. Meningococci with decreased susceptibility to penicillin (38.9 %) and one strain resistant to ciprofloxacin were identified. Forty-two percent of MenB showed the presence of at least one of the 4CMenB vaccine antigens (*fHbp*, *NHBA*, *NadA* and PorA). Our findings highlight the genetic variability of meningococci causing IMD in Croatia, especially for the serogroup B. Molecular-based characterization of meningococci is crucial to enhance IMD surveillance and to better plan national immunization programmes.

**INTRODUCTION**

Invasive meningococcal disease (IMD) is caused by *Neisseria meningitidis*, a strict human pathogen affecting mainly children ≤5 years of age, adolescents and young adults (Stephens, 2009). IMD is characterized by meningitis, sepsis and less commonly pneumonia, arthritis and pericarditis.

According to the European Centre for Disease Prevention and Control (ECDC) data, the incidence of IMD reported in European Union (EU) and European Economic Area countries in 2012 was substantially low (ECDC, 2014), with an overall notification rate of 0.68 cases per 100 000 inhabitants. IMD case fatality ranged from 8 to 15 %, and approximately 10–20 % of survivors suffered from long-term sequelae including mental retardation, motor nerve deficit, hearing loss and loss of limbs (ECDC, 2014; Stephens, 2009).

†These authors contributed equally to this work.

**Abbreviations:** 4CMenB, four-component meningococcal serogroup B vaccine; cc, clonal complex; CI PH, Croatian Institute of Public Health; ECDC, European Centre for Disease Control; EU, European Union; fHbp, factor H-binding protein; IMD, invasive meningococcal disease; MATS, multiantigen typing system; MIC, Minimum Inhibitory Concentration; MLST, multilocus sequence typing; NadA, Neisseria adhesin A; NHBA, Neisseria heparin-binding antigen; ST, sequence type; WGS, whole-genome sequencing.
In the EU area, serogroup B is the predominant cause of IMD, followed by serogroup C, whereas serogroup Y is recently increasing (Brüker et al., 2015; Törös et al., 2014). The only successful meningococcal disease prevention tool is represented by conjugate vaccines, including both meningococcal serogroup C glycoconjugate vaccines and quadrivalent conjugate vaccines against serogroups A, C, Y and W. Moreover, a novel meningococcal multicomponent vaccine, four-component meningococcal serogroup B (4CMenB; Bexsero®), has been approved in Europe, Canada, Australia and USA. Bexsero comprises three recombinant proteins [factor H-binding protein (fHbp), Neisseria heparin-binding antigen (NHBA) and Neisseria adhesin A (NadA)] and PorA protein (Giuliani et al., 2006).

In Croatia, the incidence of IMD remained quite stable, around 1 case per 100,000 inhabitants in the last decade (Bukovski & Jelic, 2014) with most of the cases due to serogroup B meningococci.

The aim of this study was to determine the molecular characteristics of a collection of meningococci causing IMD occurring in Croatia from 1 June 2009 to 31 January 2014.

METHODS

IMD surveillance system in Croatia. In Croatia, IMD is included among the notifiable infectious diseases which are reported to the Croatian National Institute of Public Health (CIPH). Clinicians immediately report every IMD suspected case to the CIPH epidemiologist. Data on laboratory-confirmed cases and N. meningitidis serogroup are also recorded. In particular, the laboratory-based surveillance system for bacterial meningitis was established in 2000 (Boras et al., 2004). The University Hospital for Infectious Diseases of Zagreb where bacterial isolates are collected is also part of the National Croatian Centre.

Patients, clinical specimens and method of laboratory diagnosis. According to the CIPH, the IMD incidence rate from 2000 to 2010 ranged from 0.8 to 1.4 per 100,000 (Klismanic et al., 2013). From June 2009 to January 2014, 50 cases of IMD were laboratory confirmed. During the study period, N. meningitidis was detected in 48 cerebrospinal fluids and 2 blood samples. Twenty-eight strains of N. meningitidis were isolated, and DNA was detected by PCR in 22 samples.

Serogroup determination was by slide agglutination test on isolated meningococci (Remel Europe); furthermore, confirmation of detection of meningococcal genomic DNA from culture-positive and culture-negative samples to determine ST, cc, and PorA protein (Giuliani et al., 2006).

METHODS

From the extracted genomic DNA using the Nxtera XT DNA sample preparation kit, and a 2×300 nt paired-end sequencing run was performed with Illumina MiSeq platform (kit v3, 600 cycles). A mean of 824,873 (∗2) paired-end reads was obtained for each sample. The reads were trimmed and de novo assembled using the software ABySS version 1.3.5 (Simpson et al., 2009). The genomic assembly generated a mean of 240 contigs >500 nt, and the estimated genome size ranges from 2.0 to 2.2 Mb. The genome sequences were uploaded to the Neisseria PubMLST database (http://pubmlst.org/neisseria/) to obtain sequence type (ST), clonal complex (cc), porA and fetA type, Bexsero vaccine antigens (fHbp, NHBA and NadA) and antibiotic resistance genes (gyrA, penA and rpoB).

PCR and Sanger sequencing were used for the remaining 28 DNAs obtained from culture-negative samples to determine ST, cc, porA and fetA (Birtles et al., 2005; Thompson et al., 2003). Briefly, for fetA gene amplification and sequencing, oligonucleotides described by Thompson et al. (2003) were used. PCR run was as follows: 94°C for 1 min, 67–52°C for 1 min (with a decrease of −0.5°C per cycle), 72°C for 2 min for 30 cycles. Additional 10 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min with a final extension of 72°C for 1 min were also included.

The nucleotide sequences were assembled using ChromasPro Version 1.15, and allele numbers were assigned by querying the PubMLST database (http://pubmlst.org/neisseria).

RESULTS

Patient and clinical samples

A total of 50 samples from IMD cases were collected; of them, 22 (44%) were confirmed by culture and PCR, whereas 28 (56%) were confirmed by PCR only.

Meningitis was the clinical manifestation in 27 IMD cases (54%); meningitis/septicaemia and septicemia were diagnosed in the remaining patients. None of the cases was fatal. Only three patients were discharged with two or more sequelae (neurological and skin necrosis). All IMD cases occurred among unvaccinated patients.

Age was available for 45 of the 50 patients: 35.5% were <5 years old, 17.7% were 5–14 years old, 17.7% were 15–24 years old and 28.9% were >24 years old. Information on gender was available for 47 IMD cases: 27 were males and 20 were females.

Serogroup distribution

Serogroup was determined in all the samples. Eighty-four percent (42/50) belonged to serogroup B; 10% (5/50), to serogroup C; 4% (2/50), to serogroup W; and 2% (1/50), to serogroup Y.

Serogroup B was the most common in each age group. Serogroup C was identified in meningococci isolated from patients aged <5 years (n=3) and >24 years old (n=2); and serogroups Y and W, from patients >24 years of age.

Antimicrobial susceptibility

All the meningococci were susceptible to ceftriaxone (MIC<90 0.002 µg ml−1) and rifampicin (MIC<90 0.012 µg ml−1), and ceftriaxone resistant, MIC>0.25 µg ml−1; ciprofloxacin resistant, MIC>0.06 µg ml−1; penicillin resistant, MIC>0.25 µg ml−1; and rifampicin resistant, MIC>0.25 µg ml−1.

Molecular characterization. Whole-genome sequencing (WGS) was performed on genomic DNA extracted from 22 meningococcal isolates, using the QIAmp DNA minikit (Qiagen). DNA libraries were prepared...
Molecular characterization

Molecular characterization (MLST, PorA VRs and FetA VR) of 47 of 50 meningococcal DNAs is summarized in Table 1. By MLST, the 40 MenB studied were associated to four main ccS, of which the most frequent was cc41/44 (20/40; 50%), followed by cc213 (8/40; 20%), cc32 (4/40; 10%) and cc269 (3/40; 7.5%). The remaining five MenB (5/40; 12.5%) belonged to cc not currently assigned.

As shown in Table 1, MenB cc41/44 were grouped into 11 STs, of which 6 were represented by multiple isolates (ST-10218, n=4; ST-1194, n=3; ST-41, n=2; ST-1403, n=2; ST-7313, n=2) and 5 STs by a single isolate (ST-110, ST-2799, ST-2827, ST-3816 and ST-10979). In eight MenB/cc213, we found the ST-3496 (n=7) and ST-8756 (n=1), respectively; in four MenB/cc32, the ST-32; and in three MenB/cc269, the ST-8554 (n=2) and ST-283 (n=1), respectively. The remaining five isolates with cc unassigned were grouped in five different STs, and three of them were novel (ST-1111, ST-11111 and ST-11108) (Table 1).

Several combinations of PorA (VR1 and VR2) were identified among MenB. Collectively, the most common PorA subtype was P1.7-2,4 (n=12), P1.22,14 (n=7) and P1.18-7,9 (n=4). Of 40 MenB studied, 12 (30%) presented PorA VR2 P1.4; 11 of them belonged to cc41/44 and 1 to cc32.

F1-5 (n=9) in cc41/44, F5-5 (n=7) in cc213, F3-3 (n=2) in cc32 and F5-2 (n=2) in cc269 were the FetA VRs most frequently observed. A new FetA variant, F1-168, was found in one isolate with a cc not yet assigned.


The two MenW were cc174, W: P1.22, 26: F3-7: ST-2977, whereas the only MenY was Y:P1.5-2,10-1:F4-1:ST-23 (cc23).

Twenty-two (19 MenB, 1 MenC, 1 MenW and 1 MenY) of the 50 isolates were further investigated for the 4CMenB vaccine antigen genes: fHbp, nhba and nadA.

The distribution of the 4CMenB vaccine antigen genes in relation to cc and serogroup among 22 Croatian meningococci is shown in Fig. 1(a–c), respectively.

fHbp1.4 was the most frequent variant in MenB cc41/44 isolates (6/19), followed by fHbp1.510 (5/19) of MenB cc41/44 and MenB/cc32 and fHbp1.10 (4/19) of MenB/cc213 (Fig. 1a). fHbp1.1 (the specific fHbp included in the 4CMenB vaccine) was found in one MenB/cc32 isolate. The fHbp variant 2 was identified in three isolates belonging to other serogroups, in particular, fHbp2.22 in MenC/cc11, fHbp2.421 in MenW/cc174 and fHbp2.25 in MenY/cc23.

As shown in Fig. 1(b), NHBA-2 (the variant included in the 4CMenB vaccine) and NHBA-112 were the most represented among MenB cc41/44; NHBA-18 (3/19) was identified in MenB/cc213; NHBA-111, in two MenB cc41/44; and NHBA-29, in MenC/cc11 and in one MenB/cc32. Furthermore, NHBA-7, 3, 6, 21, 741 and 625 were represented in one isolate each.

Of 22 isolates, 7 were found to harbour nadA gene, of which NadA-4/5 (4/7) in MenB/cc213, NadA-1 (2/7) in MenB/cc32 and NadA-2/3 (1/7) in MenC/cc11 (Fig. 1c). The remaining isolates had the nadA interrupted gene due to the insertion sequence ISI301.

Antibiotic resistance genes (gyrA, penA and rpoB) were investigated in 22 isolates. A total of seven gyrA alleles were found; by far, the most common was gyrA4 (n=11). The gyrA92 allele, with the T91I amino acid substitution, was identified in one isolate which was resistant to ciprofloxacin.

Eleven penA alleles were found, of which penA1 was the most prevalent (n=7). Finally, rpoB18 was the main rpoB allele identified in the analysed strains.

DISCUSSION

With the licensure of several vaccine formulations against meningococcal disease, data on incidence and on molecular characteristics of N. meningitidis strains circulating in European countries are needed. Little information is available for Croatia, a country with a low incidence of IMD (approximately 1 case per 100 000 inhabitants), with the highest disease burden among children (Bukovski & Jelic, 2014; Bukovski et al., 2013).

Since the year 2000, serogroup B was predominant followed by serogroup C (Boras et al., 2004). As described above, meningococci of serogroup B belonged to the electrophoretic type ET-5 complex, whereas meningococci of serogroup C belonged to the ET-37 complex, now designated as cc32 and cc11 (Boras et al., 2004).

To the best of our knowledge, this study represents the first investigation of the microbiological and molecular profiles of meningococci responsible for IMD in Croatia during the period 2009–2014. Here, we combined WGS analysis on invasive meningococcal strains with epidemiological and resistance data. Moreover, the genetic diversity of vaccine antigen genes in the newer MenB vaccine was investigated. Since the increase in the use of both WGS analysis in molecular epidemiology and the resultant data available in public databases, the need for a unified format to allow comparison of representative national and international data sets was highlighted, together with a proposed solution, by Bratcher et al. (2014).

Most IMD cases were caused by MenB strains, which also showed a greater variability compared with the other
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UA, unassigned to a cc; ND, not determined.

*New ST.
†New variant.
serogroups, with cc41/44 being prevalent, together with cc32, cc269 and cc213. In this analysis, serogroup C was mainly associated with cc11, which is likely to be one of the most invasive strains, responsible for several epidemics and outbreaks worldwide (Lucidarme et al., 2015).

The majority of meningococci were susceptible to all antimicrobials tested, with the exception of one strain resistant to ciprofloxacin; a decreased susceptibility to penicillin was also observed. Moreover, the molecular analysis of antimicrobial resistance target gene identified seven penA alleles of which penA1 was predominant among the strains with intermediate susceptibility to penicillin. Similarly, the ciprofloxacin-resistant strain showed the amino acid change in gyrA gene, which was described to be associated with the ciprofloxacin resistance pattern (Hong et al., 2013). The genetic analysis of resistant target genes was in agreement with the MIC results for resistance or reduced susceptibility to antimicrobials.

Twenty-two isolates (19 MenB, 1 MenC, 1 MenW and 1 MenY) were investigated for the 4CMenB vaccine antigens: fHbp, NHBA, NadA and PorA. Based exclusively on vaccine target gene sequence analysis, 26.3% (5/19) of MenB matched at least for one antigen (5.3% fHbp1.1, 10.5% PorA and 10.5% NHBA-2, respectively), whereas 15.7% (3/19) of the isolates were found to match for two components, PorA VR2 P1.4 and NHBA-2. Considering the cross-protection among fHbp variant family 1 and NadA-1 and 2/3 peptide, two MenB isolates might also be potentially

**Fig. 1.** Distribution of fHbp variant/subvariant (a), NHBA peptide (b) and NadA variant (c) in relation to serogroup and cc in 22 Croatian meningococci. The asterisk (*) indicates the variants included in the 4CMenB vaccine.
covered by the vaccine. The vaccine antigen fHbp1.1 was poorly represented, and it was found exclusively in cc32. Conversely, a high percentage of this vaccine antigen was identified among meningococci collected in France (21.5%) and in Germany (18.9%), as already described (Vogel et al., 2013). Besides MenB, the MenC/cc11 showed the presence of NadA 2/3 peptide. Overall, the results suggest the important role of NHBA-2 and PorA VR2 P1.4 as a factor potentially contributing to vaccine coverage especially for meningococci B cc41/44. This finding is in line with other studies reporting that a high proportion of MenB of cc41/44 is likely to be covered by the 4CMenB vaccine (Vogel et al., 2013). As expected, the nadA gene was poorly represented due to the high proportion of cc41/44 isolates. In fact, as reported in the literature (Bambini et al., 2013), the nadA gene is identified in approximately 30% of meningococci, mainly associated with cc11, cc32 and cc213, whereas it is rare or absent among those associated with cc41/44 and cc269 meningococci (Bambini et al., 2013, 2014; Lucidarme et al., 2010).

In this study, three ccs were predicted to be covered by the 4CMenB vaccine antigens: cc41/44, cc32 and cc11. In particular, the vaccine antigen fHbp1.1 was found in cc32; NHBA-2 and PorA P1.7-2,4 were both found in cc41/44 isolates, whereas NadA-3 was present in one cc11 isolate. Moreover, only one cc11 isolate harboured the NadA-3 variant. Therefore, aside from MenC, cc11 was predicted to be covered by the 4CMenB vaccine, by an fHbp gene encoding for variant 1. In contrast, none of the MenW and the only one MenY was predicted to be covered by the vaccine. The number of MenC, Y and W collected and analysed was smaller; therefore, whether the vaccine may protect against these serogroups in Croatia may be difficult to predict.

However, the lack of multiantigen typing system (MATS) relative potency measure did not permit to empirically estimate the number of isolates effectively covered by the vaccine. MATS ELISA, in fact, measures the expression and diversity of the vaccine antigens for each strain. In the UK, MATS predicted coverage for MenB strains was 70% (95% confidence interval, 55–85%) (Frosi et al., 2013). In Spain, most of the MenB strains seem to be covered by only one vaccine antigen: this might be due to the emergence of cc213 in Spain which is associated with low predicted MATS strain coverage (Abad et al., 2016). Nevertheless, a 4CMenB vaccine coverage was estimated to be 89.2% (95% confidence interval, 63.5–98.6%) in Greece mainly due to the presence of NHBA-20 among cc162 MenB strains (Tzanakaki et al., 2014). Overall, as already mentioned (Brehony et al., 2015), a limited number of antigenic variants of each vaccine antigen were represented among MenB isolates, with an association with specific serogroups and ccs. However, possible cross-protection due to other serogroups in presence of the same vaccine antigens should be evaluated.

The major limitation of this study is represented by the low number of isolates. However, they accounted for 80% of all laboratory-confirmed cases reported from 2009 to 2014; thus, they may be considered representative of the strains causing IMD in Croatia (data not shown).

Overall, the evidence gathered suggests that, although the IMD incidence rate in the country appears to be lower than the average rate reported in the EU, disease-causing strains are similar to those detected in most EU countries (ECDC, 2014; Vogel et al., 2013). Furthermore, some interesting findings were found: first, serogroup B was the most common among the isolates; second, a high genetic variability of the strains belonging to serogroup B was observed. Finally, based on the analysis of the 4CMenB vaccine antigen genes, 42% of the samples showed at least one 4CMenB vaccine encoding gene. The predominance of MenB among IMD cases highlights the need for a prevention strategy targeting this serogroup. Moreover, the identification of new variants emphasized the importance of a comprehensive molecular characterization of the strains, which is crucial to determine the antigenic profile and the clonal diversity of meningococci associated with invasive disease. Finally, detailed knowledge of the meningococcal population and vaccine antigen variants may be useful to improve public health response against this relatively rare, but extremely severe, vaccine-preventable disease.

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