Establishment of an Active Laboratory-Based Surveillance for Bacterial Meningitis in Croatia and Molecular Characterization of Neisseria meningitidis Isolates Causing Meningococcal Disease That Were Collected in the Year 2000, the First Year of Activity

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In 2000, 23 Neisseria meningitidis (meningococcal [Men]) isolates were collected in Croatia through an active laboratory-based surveillance for bacterial meningitis (17 Men serogroup B [MenB], 4 MenC, 1 MenW135, and 1 nongroupable isolate). Molecular characterization revealed a substantial level of diversity with only six isolates belonging to electrophoretic type 5 (ET-5) and ET-37 hypervirulent complexes.

Over the past decade, the annual incidence of meningococcal (Men) disease in Croatia remained stable at 0.7 to 1.5/100,000 inhabitants (2). None of the Neisseria meningitidis isolates were molecularly characterized, and no comparison with strains causing Men disease worldwide was possible. Molecular subtyping of Men has provided significant insight into the epidemiology of Men disease (19). By use of multilocus enzyme electrophoresis (MEE), hundreds of different electrophoretic types (ETs) have been identified in invasive isolates, yet fewer than 10 of them have been associated with most of the Men disease worldwide (4). Sacchi et al. demonstrated the diversity of 16S rRNA gene types (16S types) among 265 Men strains of serogroups A (MenA), B, C, and Y, and identified 16S type 4 and 16S types 12 and 13 that are associated exclusively with ET-5 and ET-37 complexes, respectively (17). Finally, pulsed-field gel electrophoresis (PFGE) was effectively used to identify MenA strains of subgroup III associated with major epidemics in Africa (1) and was a powerful aid in investigations of MenC outbreaks in the United States (15).

Currently, surveillance for bacterial meningitis in Europe is based on the reports of up to 38 European countries of results of laboratory confirmations of case notifications every year (11). In support of these efforts, the objectives of this study were (i) to design and establish an active laboratory-based surveillance for bacterial meningitis in Croatia, (ii) to prospectively collect all Men isolates cultured from patients with invasive disease during the year 2000, and (iii) to molecularly characterize Croatian Men isolates and assess their relationships to major hypervirulent clonal groups circulating in Europe and worldwide.

An active laboratory-based surveillance for bacterial meningitis was established on 1 January 2000 with 10 of 20 Croatian microbiological laboratories participating and covering 10 major cities with a total population of approximately 3 million. All Men isolates were transported in silica gel packages (14) to the Microbiology Laboratory, University Hospital for Infectious Diseases, Zagreb, Croatia, and the Centers for Disease Control and Prevention (CDC), Atlanta, Ga., and were accompanied by a surveillance form that recorded each patient’s basic demographic information and clinical presentation. A clinical case of Men disease is defined as the presence of petechial or purpuric rash with clinical signs of sepsis and/or purulent meningitis in a patient. The serogroup for all isolates was determined by standard slide agglutination serogrouping (13) and a serogroup-specific PCR that is able to identify MenA, MenB, MenC, MenY, MenW135, and MenX (10). Molecular characterization was performed by MEE (16), NheI PFGE (15), and sequencing of the 16S rRNA gene (17). Antimicrobial susceptibility testing to determine the MICs of penicillin, ceftriaxone, rifampin, ciprofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole was performed by Etest (BioDisk, Solna, Sweden).

There were 41 clinical notifications of Men disease in Croatia during 2000, for an incidence of 0.9/100,000; 26 (63%) cases were laboratory confirmed by isolation of Men from blood or cerebrospinal fluid (CSF). No clusters of cases were recorded, no carriage studies were conducted, and the male-to-female ratio was 2:1. Eleven patients presented with meningitis, six presented with Men sepsis, and nine presented with both meningitis and sepsis. The age distribution of patients for...
the 26 laboratory-confirmed cases was as follows: eight were <1 year old, six were 1 to 4 years old, three were 5 to 9 years old, five were 10 to 19 years old, and the remaining four were >40 years old. Only one patient died (4% mortality).

Eighteen of the 26 isolates were collected from CSF, 7 were collected from blood, and for one patient an isolate from both blood and CSF was available; 23 isolates were available for this study (3 were nonviable upon transport). Seventeen isolates (74%) were MenB, 4 (17%) were MenC, 1 (4%) was MenW135, and 1 was nongroupable (NG). MenB and MenC accounted for 95% of cases in Europe in the 1999-2000 epidemiological year (10), which is similar to the serogroup distribution of Croatian Men strains in 2000 (74% MenB and 17% MenC). None of the four MenC isolates were susceptible to penicillin (MICs of 0.125 μg/ml for two isolates, 0.19 μg/ml for one isolate, and 0.25 μg/ml for one isolate). All other isolates were susceptible to penicillin, ceftriaxone, chloramphenicol, and ciprofloxacin, with the exception of a single MenB strain that was resistant to rifampin (MIC, ≥32 μg/ml). Only 65% of Men isolates were susceptible to trimethoprim-sulfamethoxazole.

Molecular characterization of the 23 Men isolates revealed a substantial level of diversity. By MEE, only 2 of the 17 MenB strains belonged to the hypervirulent ET-5 complex that was previously identified as a cause of major Men disease epidemics worldwide (4) (Fig. 1); both strains had 16S type 4, which to date had been seen exclusively in strains of the ET-5 complex (17) (Fig. 2). Among the remaining 15 MenB isolates, 12 different PFGE patterns (Fig. 1) and nine different 16S types were identified (Fig. 2). Overall, excellent correlation was observed between MEE, 16S rRNA sequencing, and PFGE results: more than one PFGE pattern was seen in isolates with identical 16S types (within or outside of the ET-5 complex), but no individual PFGE pattern was associated with more than a single 16S type. Further studies are needed to correlate this high level of diversity with diversity within the porA genes to assess the feasibility of the use of outer membrane protein-based vaccines against MenB in Croatia.

All four MenC isolates belonged to the hypervirulent ET-37 complex. Two very similar PFGE patterns (89% similarity) were observed, but neither has been previously identified among several hundred serogroup C strains in the CDC PFGE database. All four had a novel 16S type (type 91), differing by only a single base from 16S type 13, the 16S type most frequently identified in MenC strains of the ET-37 complex (17). A single MenW135 isolate represented the first invasive case of Men disease ever reported in Croatia that was caused by this serogroup (3, 9).

Among the countries bordering Croatia, limited information is available on Men disease, serogroup distribution, and further characterization of Men. An even higher proportion of MenB (91%) was reported in children in Slovenia in 1993 to 1999 than in Croatia (12). By MEE, these strains were found to be very heterogeneous, and only three belonged to the ET-5 complex, a distribution very similar to that of the Croatian MenB strains. Multilocus sequence typing (MLST) identified strains of sequence type 247 (ST-247) in an outbreak investigation in Hungary in 2000 (D. Caugant, personal communication). ST-247 is closely related to ST-11, which is the typical representative of the ET-37 complex. MLST is gaining importance as a subtyping tool for Men (8), and currently over 2,000 Men STs are deposited in the MLST database (http://www.mlst.net). Somewhat more information was available for countries in Central and Eastern Europe. Between 1995 and 2000, most cases in Romania were caused by MenB (38%) (7). In the 1999-2000 epidemic year, MenB predominated in Austria (77%), Italy (81%), and Greece (58%) (11). Currently only 17 isolates (13 from Austria and 4 from Romania) have been submitted to the MLST database. Among the Austrian Men strains, two belonged to the ET-37 complex. MenB predominated in Poland in 2000 (18), and MenB became predominant (59%) (6) in the Czech Republic after 7 years of prevalence of MenC among the invasive isolates, whereas MenC predominated in the Slovak Republic with an increasing trend in the proportion of strains belonging to the ET-37 complex (74%) (5).

This is the first study addressing the molecular epidemiology of Men disease in Croatia. It showed a substantial level of diverse molecular markers among these strains, with less than a quarter of them having molecular markers typical for strains of the ET-5 and ET-37 complexes. Continuation of the surveillance will contribute to the control of Men disease in Croatia by aiding in the assessment of need and feasibility of use of outer membrane protein and conjugate vaccines for prevention and control of MenB and MenC disease, respectively.

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