Original Article

Serological and Genetic Characterization of Meningococcal Isolates in Korea

Song-Mee Bae and Yeon-Ho Kang*

Division of Bacterial Respiratory Infections, Center for Infectious Diseases, National Institute of Health, Korea Centers for Disease Control and Prevention, Seoul, Korea

(Received January 30, 2008. Accepted July 16, 2008)

SUMMARY: Meningococcal disease has been regarded as a very rare infection in Korea. Until now, there have been no reports on the serological or genetic characterization of Neisseria meningitidis isolates in Korea. This study was the first report of the serogroup, PorA VR subtype, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and antimicrobial susceptibility of N. meningitidis isolates collected from 2002 to 2003. Of 11 meningococcal isolates, serogroup Y was found to be the most frequent (nine isolates). In addition, one isolate was from serogroup B and one was from serogroup 29E. Four isolates showed a reduced sensitivity to penicillin G. However, all strains tested were susceptible to chloramphenicol, cefotaxime, ciprofloxacin, and rifampin. Among the 11 isolates, seven PorA types were identified. P1.5-1, 2-2 was the most prevalent PorA type, accounting for 55.6% of the serogroup Y isolates. In terms of PFGE patterns, nine isolates of serogroup Y were divided into three clusters, but the isolates shared a high level of PFGE pattern similarity. The serogroup Y isolates were characterized as ST-1625 (five strains) and ST-23 (four). They belonged to the ST-23 complex/Cluster A3. In this study, the ST-23 complex/Cluster A3 was prevalent, with the PorA type P1.5-1, 2-2 accounting for 55.6% of the nine serogroup Y strains. Also, we identified the ‘hypervirulent lineage’ strain such as ST-6667 of ST-41/44 complex/Lineage 3 in Korea. The results of this study show the need for comprehensive epidemiological surveillance to monitor any changes in the meningococcal disease situation so that prompt intervention can be initiated.

INTRODUCTION

Neisseria meningitidis is a major cause of bacterial meningitis and sepsisemia in children and young adults (1,2). Meningococcal disease usually occurs sporadically in developed countries, but outbreaks and large epidemics occur around the world, especially in the African ‘meningitis belt’ (3). N. meningitidis is divided into 13 serogroups based on the immunological specificity of the capsular polysaccharide (4). Among them, serogroups A, B, and C have been the main causes of most endemic meningococcal disease and of most meningococcal disease epidemics (3). However, serogroup Y and W135 meningococci have recently become more significant (4,5).

In Korea, meningococcal disease is a notifiable disease and, thus, physicians are obligated to report any confirmed case to the Korea Centers for Disease Control and Prevention. Between 1990 and 2001, 2 to 13 probable cases of meningococcal disease were reported annually. The disease has been regarded as a rare infection. During the period from 2002 to 2003, the number of reported cases of meningococcal disease in Korea dramatically increased (27 cases in 2002 and 38 cases in 2003) (6). These increases suggest that the burden of meningococcal disease must be taken seriously in this country.

Until now, there have been no reports on the serological or genetic characterization of N. meningitidis isolates in Korea. Even though there are too few isolates to represent the situation of meningococci in this country, we provide here the first report on the serogroup, PorA VR subtype, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and antimicrobial susceptibility of N. meningitidis isolates collected during the period from 2002 to 2003 in Korea.

MATERIALS AND METHODS

A total of nine N. meningitidis isolates were isolated from blood and/or cerebrospinal fluid (CSF) specimens from patients with meningococcal disease throughout Korea. Also, two isolates were obtained from a pharyngeal swab of an asymptomatic carrier who had close contact with patients between 2002 and 2003.

All strains were confirmed to be N. meningitidis by conventional methods including colony morphology on chocolate agar plates, Gram staining, oxidase tests, catalase tests, carbohydrate fermentation tests, and the API NH kit. The strains were grown on chocolate agar plates at 37°C in 5% CO₂ and stored at −70°C as suspensions in 20% glycerol (v/v) in brain heart infusion (BBL Microbiology Systems, Cockeysville, Md., USA) until further processing. Serogrouping was determined by the slide agglutination method with polyclonal antisera (Difco, Detroit, Mich., USA).

Antimicrobial susceptibilities to penicillin G, chloramphenicol, cefotaxime, ciprofloxacin, and rifampin were determined by the broth microdilution method in Muller-Hinton broth supplemented with 3% lysed horse blood according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (7). The breakpoints used in this study were those recommended by the NCCLS for Neisseria gonorrhoeae.

Amplification of the porA gene by polymerase chain reaction (PCR) and sequencing of the porA variable regions (VR1

*Corresponding author: Mailing address: Division of Bacterial Respiratory Infections, Center for Infectious Diseases, National Institute of Health, Korea Centers for Disease Control and Prevention, 5 Nokbun-Dong, Eunpyung-Ku, Seoul, 122-701, Korea. Tel: +82-2-380-2140, Fax: +82-2-385-8043, E-mail: slowpc@hanmail.net
For the amplification of porA genes encoding the VR1 and VR2 regions, two primer sets designed to be complementary to the conserved regions of the porA gene (U86 and R435 for the VR1 region; F435 and R773 for the VR2 region) were used. Sequencing was performed using a BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, Calif., USA). All nucleotide sequences for each VR were aligned with reference to both the nucleotide and the corresponding amino acid sequences through a Web-accessible PorA database (http://neisseria.org/nm/typing/pora) and defined subtype families.

The PFGE procedures used in this study were the following. Briefly, bacteria grown on chocolate agar plates were suspended in TE buffer, mixed with an equal volume of low-melting-temperature agarose (Bio-Rad Laboratories, Hercules, Calif., USA), and placed in a slot former (Bio-Rad). The resulting inserts were placed in 2 ml of lysis buffer (50 mM Tris-50 mM EDTA-1% Sarkosyl, pH 8.0) and after the addition of 10 µl of proteinase K (20 mg/ml) were incubated for 24 h at 56°C. After washing, the DNA was digested overnight with Spel (Promega, Madison, Wis., USA). Restriction fragments were subjected to electrophoresis in a 1% agarose gel in TBE buffer with a contour-clamped homogeneous electric field (CHEF DRIII; Bio-Rad). Pulse times were ramped from 1 to 25 s for 20 h at 5 V/cm. Gels were stained with ethidium bromide, and PFGE patterns were detected by UV transillumination. A dendrogram of PFGE patterns was constructed using the unweighted pair group method of average linkage (UPGMA).

MLST was performed as described by Maiden et al. (9) with some modification. MLST loci including abc (putative ABC transporter), adk (adenylate kinase), adhE (shikimate dehydrogenase), fumC (fumarate hydratase), gdh (glucose-6-phosphate), pdhC (pyruvate dehydrogenase subunit I), and pgm (phosphoglucomutase) were sequenced. Each sequence was assigned an allele number based on the previously observed allelic sequences in the Neisseria MLST database (http://neisseria.org/nm/typing/mlst). The sequence type (ST) number of each strain was determined from the profiles of the seven alleles, and the relationship of the STs with the defined clonal complex was assigned automatically.

RESULTS

During the period of 2002 to 2003, 65 cases of meningococcal disease were reported by hospitals to the Korea Centers for Disease Control and Prevention; nine of these were isolated from blood or CSF specimens of meningococcal patients from diverse locations throughout Korea (Table 1). There were no epidemiological links among the cases. Two isolates were also obtained from throat swabs of two asymptomatic carriers who were students in the same class at school. Of the 11 meningococcal isolates, serogroup Y was found most frequently (nine isolates) in this study. In addition, one isolate was from serogroup B and one from serogroup 29E.

The susceptibility to antibiotics of the 11 meningococcal strains was assessed by the broth microdilution procedure (Table 1). All 11 meningococci were found to have penicillin G MICs of 0.016 to 0.25 µl/ml. Four isolates (36.4%) showed reduced sensitivity to penicillin G; there were no resistant strains (≥2 µl/ml) detected. All strains were susceptible to chloramphenicol, cefotaxime, ciprofloxacin, and rifampin.

Serosubtypes, defined by variation in the outer membrane protein PorA, are an integral part of the characterization scheme for N. meningitidis (8). Among all 11 isolates, 11 different PorA VR sequences were identified: five in VR1 (5-1, 5-2, 5-5, 19, 21-2) and six in VR2 (2-1, 2-2, 10-1, 10-4, 14-10, 15) (Fig. 1). These amino acid sequences were grouped into seven different families. In particular, P1.5-1, 2-2 was the most prevalent PorA type, accounting for 55.6% of serogroup Y isolates.

The genetic relatedness of N. meningitidis isolates was investigated by cluster analysis using PFGE and MLST.

As shown in the dendrogram (Fig. 1), the PFGE patterns of the serogroup B and 29E isolates were divided into categories according to their isolates. Nine isolates of serogroup Y were divided into three genetic clusters (I, II, and III) based on more than 85% homology as the criterion for a cluster. However, the isolates within serogroup Y shared a high level (more than 90%) of PFGE pattern similarity and clustered tightly.

By MLST analysis, 4 STs (ST-23, ST-1625, ST-60, and ST-6667) were identified in this study (Fig. 1). ST-6667, a novel ST type which has not been found before (shown with an asterisk in Fig. 1), was assigned as a serogroup B isolate, and was resolved into the ST-41/44 complex/Lineage 3. ST-60 (ST-60 complex) was assigned as a serogroup 29E isolate. The serogroup Y isolates were characterized as ST-1625 (five strains) and ST-23 (four). They belonged to the ST-23 complex/Cluster A3. In this study, there was a prevalent ST-23 complex/Cluster A3 with PorA type P1.5-1, 2-2 accounting

### Table 1. Meningococcal strains and their relevant characteristics in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (y)</th>
<th>Site</th>
<th>Place isolated</th>
<th>Clinical disease</th>
<th>Serogroup</th>
<th>PEN (µg/ml)</th>
<th>CHL (µg/ml)</th>
<th>CEF (µg/ml)</th>
<th>CIP (µg/ml)</th>
<th>RIM (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNIH02-1</td>
<td>0</td>
<td>CSF</td>
<td>Gyeonggi-do</td>
<td>Meningitis</td>
<td>B</td>
<td>0.25</td>
<td>0.25</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNIH03-1</td>
<td>9</td>
<td>CSF</td>
<td>Chungcheongnam-do</td>
<td>Meningitis</td>
<td>Y</td>
<td>0.125</td>
<td>0.5</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNIH03-2</td>
<td>39</td>
<td>CSF</td>
<td>Chungcheongnam-do</td>
<td>Meningitis</td>
<td>Y</td>
<td>0.25</td>
<td>0.5</td>
<td>&lt;0.0078</td>
<td>0.03</td>
<td>0.016</td>
</tr>
<tr>
<td>KNIH03-3</td>
<td>62</td>
<td>Blood</td>
<td>Daegu Metropolitan City</td>
<td>Meningitis</td>
<td>29E</td>
<td>0.03</td>
<td>0.5</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNIH03-7</td>
<td>3</td>
<td>CSF</td>
<td>Incheon Metropolitan City</td>
<td>Meningitis</td>
<td>Y</td>
<td>0.06</td>
<td>0.125</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNIH03-8</td>
<td>9</td>
<td>CSF/blood</td>
<td>Gyeonggi province</td>
<td>Meningitis</td>
<td>Y</td>
<td>0.016</td>
<td>0.125</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNIH03-9</td>
<td>43</td>
<td>Blood</td>
<td>Seoul Metropolitan Government</td>
<td>Meningitis</td>
<td>Y</td>
<td>0.0025</td>
<td>1</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNM-1</td>
<td>22</td>
<td>CSF</td>
<td>Seoul Metropolitan Government</td>
<td>Meningitis</td>
<td>Y</td>
<td>0.25</td>
<td>0.5</td>
<td>0.063</td>
<td>0.063</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNM-2</td>
<td>30</td>
<td>Blood</td>
<td>Daegu Metropolitan City</td>
<td>Bacteremia</td>
<td>Y</td>
<td>0.016</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNIH03-3</td>
<td>16</td>
<td>Throat</td>
<td>Jeollanam-do</td>
<td>Asymptomatic</td>
<td>Y</td>
<td>0.03</td>
<td>0.5</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
<tr>
<td>KNIH03-4</td>
<td>16</td>
<td>Throat</td>
<td>Jeollanam-do</td>
<td>Asymptomatic</td>
<td>Y</td>
<td>0.016</td>
<td>0.5</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
<td>&lt;0.0078</td>
</tr>
</tbody>
</table>

PEN, penicillin; CHL, chloramphenicol; CEF, cefotaxime; CIP, ciprofloxacin; RIM, rifampin; CSF, cerebrospinal fluid.
Meningococcal disease is a communicable disease that must be reported by law (Group III) in Korea. The number of reported cases of meningococcal disease in Korea has been very low, and only a few cases have been confirmed by bacterial culture. For these reasons, there are few data on the serological and genetic characteristics of meningococcal disease. However, since the 1980s, an emergence of meningococcal isolates with reduced susceptibility to penicillin G has been detected in many countries including England, France, Spain, and South Africa (5,9). This study is the first report of the serogroup, Penicillin G has been an effective antibiotic for the treatment of meningococcal disease in the United States and Japan since the 1990s (2,13). Particular, there were no meningococcal strains with high-level resistance to penicillin (MIC, ≥2 μg/ml) due to beta-lactamase production (data not shown). While penicillin G is still the antibiotic of choice for meningococcal disease, the emergence of meningococcal isolates with reduced susceptibility to penicillin G is of particular concern in regard to the management of patients with meningococcal disease in Korea.

**DISCUSSION**

Limited data on the serogroups of N. meningitidis in Korea were reported in studies of the Korean army. Between 1990 and 1991, there were four cases of meningococcal infection among the Korean army recruits at Nonsan army training center, and in two patients, serogroup C strains were isolated from the blood or the CSF (11). Also, it was reported that a total of 12 cases of meningococcal disease occurred in the Korean Army from August 2000 to July 2001. Of these cases, one case in serogroup A and three cases in serogroup C were identified by PCR (12).

In this study, we found that the majority (81.8%) of meningococci in our collection of isolates was in serogroup Y and the remaining were in serogroup B and 29E. However, these findings may not reflect the general situation of meningococcal disease in the Korean population because too few isolates were analyzed, and the study was short term. Although serogroup Y strains have not been associated with large epidemics, the importance of serogroup Y strains has increased in the United States and Japan since the 1990s (2,13). Particularly, serogroup Y strains apparently caused a small meningococcal disease outbreak in central Taiwan in 2001 (14).

Penicillin G has been an effective antibiotic for the treatment of meningococcal disease. However, since the 1980s, an increasing number of meningococcal isolates with reduced susceptibility to penicillin G have been detected in many countries including England, France, Spain, and South Africa (5,9). In this study, four meningococci with reduced susceptibility to penicillin had an MIC of penicillin G of 0.125 - 0.5 μg/ml. However, there were no meningococcal strains with high-level resistance to penicillin (MIC, ≥2 μg/ml) due to beta-lactamase production (data not shown). While penicillin G is still the antibiotic of choice for meningococcal disease, the emergence of meningococcal isolates with reduced susceptibility to penicillin G is of particular concern in regard to the management of patients with meningococcal disease in Korea.

Serosubtyping, defined by variation in the outer membrane protein PorA, is important for epidemiologic monitoring and protein-based vaccine development for N. meningitidis (16). Historically, serosubtyping of meningococci has been performed using a monoclonal antibody (MAb)-based system, which recognizes antigenic differences in the PorA. However, this procedure was not comprehensive, and a large proportion of isolates could not be typed by this approach. These results were due to the fact that variants could not be recognized by MAbs, as well as to the lack of PorA expression. Genotypic analysis of the VR1 and VR2 sequences of the porA gene provides complete identification and overcomes prior limitations (16,17). In the present study, the genosubtyping of the porA gene was characterized on the basis of the PorA VR1 and VR2 amino acid sequences derived from the nucleotide sequence data. Our genosubtyping data revealed the diversity of the PorA types of N. meningitidis. In particular, there were significant differences in the PorA types among other serogroup isolates. Of note was the finding that the PorA types in the serogroup Y isolates were relatively uniform. Serogroup Y isolates expressing P1.5-1, 2-2 PorA were predominant, accounting for 55.6% of all group Y isolates.

Molecular typing methods for N. meningitidis have been used for epidemiological investigation of meningococcal disease (15). First, the genetic diversity of N. meningitidis was investigated by PFGE in this study. We found that the isolates in serogroup Y shared higher levels of PFGE pattern similarity than those in the other serogroups investigated, including B and 29E. The dendrogram analysis revealed that nine serogroup Y isolates could be further differentiated into three clusters with genetic heterogeneity. Even including the serogroup Y isolates from sporadic cases, the isolates within the same cluster shared high levels of PFGE pattern similarity (>90%).

Second, MLST analysis showed that all serogroup Y isolates in our collection from 2002-2003 were found to be ST-
23 and ST-1625, which belong to the ST-23 complex/Cluster A3. In particular, it was reported that ST-23 was the cause of a small-scale outbreak of meningococcal disease in Central Taiwan in 2001 (14) and was the dominant type in Japan (13). ST-1625 differed from ST-23 only at the \textit{fumC} locus (with allele 9 in ST-23 and allele 14 in ST-1625). Unlike ST-23, ST-1625 was not detected in Taiwan and Japan, implying that the situation in Korea might be slightly different. Also, we identified the 'hypervirulent lineage' strain such as ST-6667 of the ST-41/44 complex/Lineage 3 in Korea.

This is the first report on meningococci isolated in Korea that characterizes them by serogroup, \textit{PorA} subtype, PFGE, MLST analysis, and antibiotic susceptibility. To provide greater understanding of more isolates, it is necessary to improve the laboratory culture methods for confirmed cases of meningococcal disease. Our results show the need for comprehensive epidemiological surveillance to monitor any changes in the meningococcal disease situation so that prompt intervention can be initiated.

REFERENCES