Characterization of *Neisseria meningitidis* Isolates from Recent Outbreaks in Ethiopia and Comparison with Those Recovered during the Epidemic of 1988 to 1989

Gunnstein Norheim,1 Einar Rosenqvist,1 Abraham Aseffa,2 Mohammed Ahmed Yassin,3,4 Getahun Mengistu,5,6 Afework Kassu,5 Dereje Fikremariam,7 Wegenie Tamire,7 E. Arne Hoiby,1 Tsegaye Abele,8 Degu Berhanu,2 Yarid Merid,4 Morten Harboe,2,9 and Dominique A. Caugant1,10*

Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway1; Armauer Hansen Research Institute, Addis Ababa, Ethiopia2; Liverpool School of Tropical Medicine, Liverpool, United Kingdom3; Southern Nations, Nationalities, and Peoples’ Region Health Bureau, Awassa, Ethiopia4; Gondar College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia5; Department of Internal Medicine, Faculty of Medicine, Addis Ababa University, Addis Ababa, Ethiopia6; Sidamo Regional Hospital, Yirgalem, Ethiopia7; North Gondar Zone Health Bureau, Gondar, Ethiopia8; Institute of Immunology, University of Oslo and Rikshospitalet University Hospital, Oslo, Norway9; and Department of Oral Biology, University of Oslo, Oslo, Norway10

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The objectives of this study were to collect and characterize epidemic meningococcal isolates from Ethiopia from 2002 to 2003 and to compare them to 21 strains recovered during the previous large epidemic of 1988 to 1989. Ninety-five patients in all age groups with clinical signs of meningitis and a turbid cerebrospinal fluid (CSF) sample were included in the study of isolates from 2002 to 2003. Seventy-one patients (74.7%) were

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Further genetic analyses have shown that subgroup III strains may also differ at several other loci different from those analyzed by MLST as, e.g., loci encoding expressed surface epitopes (57). The replacement of ST-5 by ST-7 among subgroup III strains in the African continent in the mid-1990s reflects a significant genetic change (34), and there is interest in finding the immunologically relevant surface-exposed antigens that might have driven this shift (4).

Meningococcal meningitis in both endemic and epidemic forms has affected Ethiopia for over a century (7). Outbreaks and epidemics have been reported in 1935, the 1940s, the

Serogroup A *Neisseria meningitidis* is responsible for recurring epidemics of bacterial meningitis in the African meningitis belt (26). Although epidemics caused by serogroup W135 have recently arisen (54), most of the cases in the region are still caused by serogroup A meningococci (http://www.who.int/csr

Minimal multilocus sequence typing (MLST) analyses of the Ethiopian ST-5 and ST-7 strains also differed in their repertoire of lipooligosaccharides and Opa proteins between the old and the recent strains. PCR analysis of the nine *lgt* genes revealed the presence of the *lgtAHFG* genes in both old and recent strains; *lgtB* was present in only some of the strains, but no correlation with sequence type was observed. Further analysis showed that in addition to their *pgm* alleles, the Ethiopian ST-5 and ST-7 strains also differed in their *tbpB*, *opa*, *fetA*, and *lgtA* genes. The occurrence of new antigenic structures in strains sharing the same serogroup, PorA, and PorB may help explain the replacement of ST-5 by ST-7 in the African meningitis belt.

Sulfonamide, were serotyped as A:4/21:P1.20,9, and belonged to sequence type 7 (ST-7). The strains from 1988 to 1989 were also equally susceptible and were characterized as A:4/21:P1.20,9, but they belonged to ST-5. Antigenic characterization of the strains revealed differences in the repertoire of lipooligosaccharides and Opa proteins between the old and the recent strains. PCR analysis of the nine *lgt* genes revealed the presence of the *lgtAHFG* genes in both old and recent strains; *lgtB* was present in only some of the strains, but no correlation with sequence type was observed. Further analysis showed that in addition to their *pgm* alleles, the Ethiopian ST-5 and ST-7 strains also differed in their *tbpB*, *opa*, *fetA*, and *lgtA* genes. The occurrence of new antigenic structures in strains sharing the same serogroup, PorA, and PorB may help explain the replacement of ST-5 by ST-7 in the African meningitis belt.

DNA (29), epidemic serogroup A strains of *N. meningitidis* mainly express a single PorA subtype that changes only slowly over time (44).

Using multilocus sequence typing (MLST), strains of subgroup III were found to belong to two main sequence types (STs), either ST-5 or ST-7, which differ in MLST solely by their allele at the *pgm* gene (31). The ST-5 clone was introduced in Africa in 1987; between 1988 and 1999, it reached all the countries of the meningitis belt, where it was responsible for numerous outbreaks. ST-7 was identified for the first time in sub-Saharan Africa in 1996, but since 2002, mostly ST-7 strains have been isolated in the region (35).

Further genetic analyses have shown that subgroup III strains may also differ at several other loci different from those analyzed by MLST as, e.g., loci encoding expressed surface epitopes (57). The replacement of ST-5 by ST-7 among subgroup III strains in the African continent in the mid-1990s reflects a significant genetic change (34), and there is interest in finding the immunologically relevant surface-exposed antigens that might have driven this shift (4).
The objectives of this study were to collect meningococcal strains from Ethiopia from 2002 to 2003, to characterize their antibiotic susceptibilities, and to study their genetic and antigenic variation, and to study their antibiotic susceptibility pattern. These strains were compared to strains isolated during the epidemic of 1988 to 1989 (19). Specifically, we studied these recent and older strains for variations in genes encoding the outer membrane (OM) proteins NadA, FetA, and TbpB and in genes associated with lipooligosaccharide (LOS) biosynthesis. NadA, FetA, and TbpB are surface-exposed phase-variable outer membrane proteins known to exert variation among meningococci and to induce antibodies following meningococcal disease (11, 29, 47). Our study revealed that all strains collected from patients in 2002 and 2003 were very homogenous and belonged to ST-7. The replacement of ST-5 by ST-7 occurred in Ethiopia between 1995 and 2000 and was accompanied by changes in _tbpB_, _opa_, _fetA_, and _lgtA_ alleles in these strains.

**TABLE 1. Officially reported numbers of meningitis cases in Ethiopia and study areas from 2000 to 2003**

<table>
<thead>
<tr>
<th>Yr</th>
<th>Whole country&lt;sup&gt;a&lt;/sup&gt;</th>
<th>North Gondar Zone, Amhara region&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sidama and Gedio Zones, SNNPR&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases</td>
<td>No. of deaths</td>
<td>CFR (%)</td>
</tr>
<tr>
<td>2000</td>
<td>855</td>
<td>19</td>
<td>2.2</td>
</tr>
<tr>
<td>2001</td>
<td>6,266</td>
<td>311</td>
<td>5.0</td>
</tr>
<tr>
<td>2002</td>
<td>2,329</td>
<td>118</td>
<td>5.1</td>
</tr>
<tr>
<td>2003</td>
<td>3,540</td>
<td>166</td>
<td>4.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data source, http://www.who.int/csr/don.

<sup>b</sup> Data sources, reference 33 and North Gondar Zone Health Bureau. The target population for vaccination was defined as those between 2 and 30 years of age in the affected subarea.

<sup>c</sup> Data sources, SNNPR Regional Health Bureau, Central Statistical Authority, Addis Ababa, Ethiopia, and ORC Macro, Calverton, MD.

<sup>d</sup> CFR is defined as the number of deaths attributed to meningitis per number of patients with meningitis.

<sup>e</sup> No. vacc, number of individuals reported as vaccinated with serogroup A and serogroup C meningococcal polysaccharide vaccine as the epidemic was evolving.

1950s, 1964, 1976 to 1977, 1981 to 1983 (18), and 1988 to 1989 (19). Prior to 1988, the majority of epidemic cases occurred in the north, the northwest, and parts of the central regions of Ethiopia, which lie within the eastern end of the traditional meningitis belt (26). After the devastating epidemic of 1988 to 1989, however, this pattern changed, and the whole country has been affected by outbreaks (46), although increased awareness could also contribute to this observation. Epidemics were also reported in Ethiopia in the years 2001 to 2003. The number of cases and case fatality rates (CFRs) from 2000 to 2003 are given in Table 1. While the epidemics in 1981 and 1988 to 1989 struck with the magnitude of 40,000 to 50,000 cases (19, 46), these recent epidemics were much smaller; most cases occurred in the Amhara region and the Southern Nations, Nationalities, and Peoples’ Region (SNNPR), respectively (http://www.who.int/csr/don) (Fig. 1).

The case definition for bacterial meningitis was made according to World Health Organization guidelines (53). Clinical data, history, information about meningococcal vaccination status, and other relevant parameters were entered into a case record form. Reports of sequelae and deaths attributed to the meningitis episode were only those observed during the admission period in the hospital, 160 km west of Gondar, and to health centers in remote villages. Patients were also admitted to Metema hospital, 160 km west of Gondar, and to health centers in remote villages.

**FIG. 1.** Map of Ethiopia, with cities of collaborating institutions indicated. The figure was prepared using ArcView 9.1 software (ESRI, Redlands, CA) and geographic data available from the European Joint Research Centre Digital Map Archive (http://dma.jrc.it).
hospitals. Lumbar puncture for CSF sampling was carried out as part of routine procedures, according to the decision of the doctor, and antibiotic treatment was started immediately thereafter, according to the treatment protocol of the respective institutions. Turbid CSF samples, remaining after local laboratory tests were performed, were collected in sterile test tubes and split into three aliquots. These were analyzed at (i) the microbiology laboratories at Gondar Medical Hospital or SNNPR Health Bureau in Awassa, (ii) the Armauer Hansen Research Institute (AHRI), and (iii) the Norwegian Institute of Public Health (NIPH), respectively, to maximize laboratory confirmation of the cases and for quality control of the laboratory procedures for culture in Ethiopia.

CSF samples and bacterial isolates. Each aliquot of turbid CSF was inoculated into a Trans-Isolate (T-I) medium (3). Following transport to laboratories in Gondar and Awassa within 24 h, the vials were vented and incubated as described previously (41). T-I medium vial 1 was cultured in either Gondar or Awassa on Thayer Martin agar medium plates with a VCNT selective supplement (lincosamine [0.5 μg/ml], colimycin [7.5 μg/ml], amphotericin B [1.0 μg/ml], and trimethoprim [5.0 μg/ml]) (NIPH). N. meningitidis isolates were identified by standard procedures (41) and serogrouped with antisera (Murex Biotech Ltd., Dartford, United Kingdom). Pure colonies were harvested into Greaves’ solution (41) and frozen at −70°C. The remaining liquid phases of the culture-negative T-I vials at NIPH were boiled and stored frozen at −70°C for PCR analyses.

In addition, 21 strains collected in Addis Ababa and the town of Zewa (170 km to the west of Addis Ababa) in Ethiopia in 1989 (19) and 3 strains collected in 2000 to 2001 in Ethiopia from the strain collection of the WHO Collaborating Centre for Reference and Research on Meningococci, Oslo, Norway, were included in the study for comparison (see Table 5). These strains had already been assigned to subgroup III based on the MLEE method (8). Strains from South Africa, Kenya, and the United Kingdom, while vials 2 and 3 were transported as soon as possible to AHRI and NIPH, respectively, for culture on either VCNT agar medium plates (AHRI) or chocolate agar plates with an LCAT selective supplement (lincomycin [0.5 μg/ml], colimycin [7.5 μg/ml], amphotericin B [1.0 μg/ml], and trimethoprim [5.0 μg/ml]) (NIPH). N. meningitidis isolates were identified by standard procedures (41) and serogrouped with antisera (Murex Biotech Ltd., Dartford, United Kingdom). Pure colonies were harvested into Greaves’ solution (41) and frozen at −70°C. The remaining liquid phases of the culture-negative T-I vials at NIPH were boiled and stored frozen at −70°C for PCR analyses.

The typability of the 64 N. meningitidis strains from Ethiopia (21 from Gondar and 43 from Awassa) (41) was determined by dot blot with whole-cell preparations, as described previously (52). We amplified the porA gene from each of the seven genes and genotyping of the encoded PorA protein were assigned genosubtype names according to the 21 currently available for porA sequences on this website (http://pubmlst.org/mlst). The porA gene was amplified using primers OTG6687 and OTG6689 (27). Following purification of the porA PCR product using the QIAquick PCR purification kit (QIAGEN), the PCR products were obtained by digestion of the purified porA PCR product using the restriction endonucleases ApoI and Spol (New England Biolabs Inc., Beverly, MA) according to the manufacturer’s instructions. The fragments were thereafter separated on 2% agarose gels, stained with ethidium bromide, and compared with band patterns of similarly digested porA PCR products from a control strain, Z1054 (porA allele 1). The band patterns with restriction enzymes ApoI, HincII, and Spol were analyzed using the software program GeneMarker (version 2.0; NEBcutter2). The porA gene of strains with different restriction fragment patterns was sequenced using primers 3/H11032 and 1/H11032 (27) and sequenced using primers OpaB1 (5′-TAT CCG TGG TGG CGT C-3′) and OpaB2 (5′-TAT CGG TGT GCC CGT C-3′). The sizes of PCR products and macrorestriction fragments were determined using a 1-kb DNA ladder molecular weight marker (Invitrogen, Carlsbad, CA). MWG-Biotech, Ebersberg, Germany, synthesized all primers.

PCR for diagnosis of culture-negative CSF samples. To detect meningococcal DNA in CSF, the porA gene was amplified using a nested PCR, as previously described (9), on a boiled liquid fraction of the inoculated T-I medium. CSF samples that were inconclusive due to inhibitors of the polymerase were restested after DNA purification with a QIAamp DNA mini kit (QIAGEN Inc., Valencia, CA). The amplified fragment of the porA gene was sequenced as described above. The deduced amino acid sequences of variable regions (VRs) 1 and 2 of the encoded PorA protein were assigned genosubtype names according to the N. meningitidis PorA variable region database (http://neisseria.org/nm/typing/pora).

PCR amplification of the orf-2 gene, encoding the N. meningitidis serogroup A polysaccharide capsule, was done as described previously (45) for the CSF samples shown to contain the porA gene.

Characterization of the strains. The nalA gene was amplified using primers and PCR conditions as described previously (11). Similarly, the meningococcal acetyltransferase genes lgtE to lgtH and lgtZ were amplified as described previously (56). Selected PCR products of the nalA promoter region, lgtE, lgtF, lgtG, and lgtZ were sequenced as described above. Additional primers were used for sequencing of lgtF (lgtF_M6 [5′-AGC GTT TCC AAC ACG AC-3′] and P6 [5′]) and lgtH (lgtH_M1 [5′-CGC GCT ATT TGA AGA TGA TG-3′] and P26 [56]). The tetA gene was amplified as described previously (47), and following sequencing of the variable region, the deduced amino acid sequences were assigned genosubtype names according to the N. meningitidis TetA variable region database (http://neisseria.org/nm/typing/teta). Analyses were based on the alignment of the ~410-nucleotide-long sequences with the 81 currently available for tetA sequences on this website. The tpbB gene was amplified using primers OTG6687 and OTG6689 (27). Following purification of the tpbB PCR product using the QIAquick PCR purification kit (QIAGEN), the PCR products were obtained by digestion of the purified tpbB PCR product using the restriction endonucleases ApoI and Spol (New England Biolabs Inc., Beverly, MA) according to the manufacturer’s instructions. The fragments were thereafter separated on 2% agarose gels, stained with ethidium bromide, and compared with band patterns of similarly digested tpbB PCR products from a control strain, Z1054 (tpbB allele 1). The band patterns with restriction enzymes ApoI, HincII, and Spol were analyzed using the software program GeneMarker (version 2.0; NEBcutter2). The tpbB gene of strains with different restriction fragment patterns was sequenced using primers 3/Met2 and 1/O1641 (29) for old strains and T55_F (5′-TGT TGA GTG CTT TGC TGG GC-3′) and T55_R (5′-TCC CCG GAA AAA GCA CTA TA-3′) for recent strains. The opdB gene was amplified using primers O3561 and O3562 (20) and sequenced using primers OpaB1 (5′-ATT CGG TGT GCC CGT C-3′) and OpaB2 (5′-AAT GTC GGG TGT CGC GC-3′). The sizes of PCR products and macrorestriction fragments were determined using a 1-kb DNA ladder molecular weight marker (Invitrogen, Carlsbad, CA). MWG-Biotech, Ebersberg, Germany, synthesized all primers.

Statistical methods. The clinical, phenotypic, and genetic strain data were analyzed using SPSS ver. 12.0.2 for Windows (SPSS Inc., Chicago, IL). The Fisher’s exact test was used to analyze the differences in proportions of the various characteristics between patients confirmed or not confirmed as meningococcal cases (Table 2), proportions of lgtB-positive strains within the different LOS immunotypes, and dot blot data (Table 3). The non-parametric Mann-Whitney U test was used for comparisons of means of days of transport in T-I medium, ages of the patients, and duration of illness and stay at the hospital.

Ethical clearance. The study obtained ethical clearance from the AHRi/All Africa Leprosy TB and Rehabilitation Training Center Ethical Clearance Committee, the National Ethical Review Committee (Ethiopian Science and Technology Commission), and the Norwegian Regional Committee for Medical Research Ethics in Western Norway (REK III). Informed written consent was obtained from patients (above 18 years of age) or their parents or guardians (for those patients below 18 years of age or with a lack of consciousness) before enrollment in the study.

Nucleotide sequence accession numbers. The sequences of the new alleles for tpbB, lgtA, and lgtZ (two alleles) have been deposited in GenBank under the accession numbers DQ355978, DQ296151, and DQ296152 and DQ296153, respectively.

RESULTS

Patients. Ninety-five patients between 6 months and 50 years of age were included in this study from April 2002 to June 2003 on the basis of clinical signs and macroscopic appearance of

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their CSF samples. Twenty-three patients were from Gondar, mainly in 2002, and 72 patients were from the SNNPR, mainly in 2003. The patients’ demographic characteristics, clinical findings, and the hospital laboratory investigation findings are shown in Table 2.

**Laboratory confirmation of meningococcal meningitis.** Forty cases were confirmed by culture of *N. meningitidis* from CSF in at least one of the study laboratories. Most isolates were obtained from the SNNPR in 2003. In the current setting of hospitals with mobile study teams, meningococci from clinical CSF samples were able to survive in T-I medium for up to 67 days. The mean time between CSF inoculation in T-I medium in Ethiopia and isolation of meningococci in Norway (34.1 days; 95% confidence interval [CI], 25.8 to 42.5) was not statistically different (*P* = 0.840) from the mean time between inoculation and cultivation for those media that were culture negative (37.7 days; 95% CI, 30.0 to 45.4).

**TABLE 2.** Demographic characteristics, clinical and laboratory findings at hospital admission, and end results for the 95 patients included in the study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value for groupa</th>
<th>All patients</th>
<th><em>N. meningitidis</em> meningitisa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North Gondar Zone</td>
<td>23/95 (24)</td>
<td>19/71 (27)</td>
<td></td>
</tr>
<tr>
<td>Sidama and Gedio Zones</td>
<td>72/95 (76)</td>
<td>52/71 (73)</td>
<td></td>
</tr>
<tr>
<td>Yr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>51/95 (54)</td>
<td>38/71 (54)</td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>44/95 (46)</td>
<td>33/71 (46)</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
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<tr>
<td>0.5–&lt;2</td>
<td>7/93 (8)</td>
<td>3/70 (4)</td>
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<tr>
<td>2–&lt;6</td>
<td>12/93 (13)</td>
<td>10/70 (14)</td>
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<tr>
<td>6–&lt;15</td>
<td>29/93 (31)</td>
<td>25/70 (36)</td>
<td></td>
</tr>
<tr>
<td>15–&lt;20</td>
<td>15/93 (16)</td>
<td>12/70 (17)</td>
<td></td>
</tr>
<tr>
<td>≥20</td>
<td>30/93 (32)</td>
<td>20/70 (29)</td>
<td></td>
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<tr>
<td>Allb</td>
<td>160/136–18–3, 14</td>
<td>15.0 (12.6–17.5), 14</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>54/93 (58)</td>
<td>38/70 (54)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>39/93 (42)</td>
<td>32/70 (46)</td>
<td></td>
</tr>
<tr>
<td>Preadmission history</td>
<td>10/90 (11)</td>
<td>6/67 (9)</td>
<td></td>
</tr>
<tr>
<td>Antibiotics given before admission</td>
<td>15/90 (17)</td>
<td>11/67 (16)</td>
<td></td>
</tr>
<tr>
<td>Previously vaccinated with serogroup A polysaccharide vaccine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Days of illness from first symptoms to examination at hospital (n = 92; range, 0–9)b</td>
<td>2.9 (2.6–3.3), 3</td>
<td>2.9 (2.5–3.3), 2</td>
<td></td>
</tr>
<tr>
<td>Clinical and laboratory findings at hospital admission</td>
<td></td>
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<tr>
<td>Nuchal rigidity</td>
<td>89/89 (100)</td>
<td>66/66 (100)</td>
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</tr>
<tr>
<td>Back rigidity</td>
<td>34/58 (59)</td>
<td>23/41 (56)</td>
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<tr>
<td>Ecchymoses</td>
<td>7/71 (10)</td>
<td>5/56 (9)</td>
<td></td>
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<tr>
<td>Petechiae, ≥10</td>
<td>15/39 (38)</td>
<td>11/28 (39)</td>
<td></td>
</tr>
<tr>
<td>Seizures</td>
<td>12/91 (13)</td>
<td>8/66 (12)</td>
<td></td>
</tr>
<tr>
<td>Shock</td>
<td>4/90 (4)</td>
<td>2/67 (3)</td>
<td></td>
</tr>
<tr>
<td>Coma</td>
<td>14/91 (15)</td>
<td>7/68 (10)</td>
<td></td>
</tr>
<tr>
<td>End resultd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>11/95 (12)</td>
<td>3/71 (4)</td>
<td></td>
</tr>
<tr>
<td>Sequelae</td>
<td>5/95 (5)</td>
<td>2/71 (3)</td>
<td></td>
</tr>
<tr>
<td>Days of stay at hospital (n = 47; range, 0–15)b</td>
<td>6.5 (5.5–7.4), 7</td>
<td>6.3 (5.2–7.3), 7</td>
<td></td>
</tr>
</tbody>
</table>

a Except where otherwise noted, values are no. of patients with characteristic/total no. of patients for whom data were available (% of reported cases).

b Cases of *N. meningitidis* meningitis confirmed by either culture or PCR of CSF (n = 71).

c As observed during hospital admission.

d Values are means (95% CI), medians.

The culture-negative CSF samples from the remaining 55 patients were further tested by PCR. Of these samples, 31 were positive (56%) and 24 were negative (44%) in the nested porA PCR. Thus, in total, 71 patients (74.7%) had laboratory-confirmed meningococcal meningitis. Except for one patient confirmed as having *Haemophilus influenzae* serotype b infection by culture, the etiological agents in the CSF samples of the remaining 23 patients were not determined.

**Comparison of the patients confirmed as having meningococcal meningitis with the other meningitis patients.** While 11 of the 95 meningitis cases (11.6%) resulted in death during hospital stay, only 3 of the fatal cases occurred in patients confirmed as having *N. meningitidis* in CSF by culture or PCR, resulting in a meningococcal meningitis-specific CFR of 4.2%. This contrasts with the significantly higher CFR among patients with nonmeningococcal meningitis (*P* = 0.0001) (Table 2). Three patients were reported as having sequelae during the hospital stay that could be attributed to the meningitis episode. Two of them, confirmed as meningococcal meningitis cases, had hearing abnormality; the third one suffered paresis of eye muscles. At least two more patients developed sequelae attributable to the meningitis episode after discharge from the hospital. These were identified among those patients contacted up to 1 year after the onset of disease during late-convalescent-phase blood sample collection. Both patients were confirmed as having *N. meningitidis* by PCR; one had hearing impairment, and the other had central nervous system complications. There was a significantly higher frequency of coma in patients with nonmeningococcal meningitis (*P* = 0.021) (Table 2). No other factors were significantly different between patients with or without demonstrable *N. meningitidis* in their CSF (Table 2).

**TABLE 3.** Phenotypic characteristics of 64 strains isolated in Ethiopia from 1988 to 2003

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. (%) of positive reactions for time periodc</th>
<th>lgtB positivea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2000–2003 (n = 43)</td>
<td>1988–1989 (n = 21)</td>
</tr>
<tr>
<td>OpA</td>
<td>36/84 (42)</td>
<td>10/48</td>
</tr>
<tr>
<td>Opa type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>7 (16)</td>
<td>4 (19)</td>
</tr>
<tr>
<td>5f</td>
<td>13 (30)</td>
<td>4 (19)</td>
</tr>
<tr>
<td>5h</td>
<td>3 (7)</td>
<td>0</td>
</tr>
<tr>
<td>5i</td>
<td>0</td>
<td>2 (10)</td>
</tr>
<tr>
<td>5af</td>
<td>5 (12)</td>
<td>6 (29)</td>
</tr>
<tr>
<td>5ah</td>
<td>1 (2)</td>
<td>0</td>
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<tr>
<td>5fh</td>
<td>3 (7)</td>
<td>0</td>
</tr>
<tr>
<td>5ai</td>
<td>0</td>
<td>2 (10)</td>
</tr>
<tr>
<td>NadaA</td>
<td>10 (23)</td>
<td>8 (38)</td>
</tr>
<tr>
<td>LOS types</td>
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</tr>
<tr>
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</table>

a Only clearly positive reactions are reported as positive. Values in parentheses are percentages. Identities of MAbs used in dot blot testing of whole cells are listed in Materials and Methods.

b Proportion of isolates of the different LOS immunotypes with positive reaction in lgtB-specific PCR. Values in parentheses are percentages.
Vaccination status was self-reported by the patients, and data should thus not be used as an indication of vaccine efficacy.

Characterization of meningococcal strains and DNA from patients from 2002 to 2003. All 40 strains were serogroup A, serotype 4/21, and serosubtype P1.20,9. When tested by MLST, they were assigned to ST-7, belonging to the ST-5 complex/subgroup III. PorA VR typing of the gene product from the 31 porA PCR-positive CSF samples revealed that all of them had meningococcal DNA from a P1.20,9 strain. Further PCR done on the same 31 CSF samples showed that 21 of the samples were also positive for the serogroup A capsule gene orf-2. Thus, all 71 patients had been infected by serosubtype P1.20,9 strains; 61 of these samples were also confirmed as being serogroup A strains.

Testing of antibiotic susceptibility revealed full sensitivity to penicillin G, ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, and rifampin, but all 40 strains were resistant to sulfamethoxazole (all with MICs ≥ 256 mg/liter) (10).

Phenotypic comparison of the strains from 2000 to 2003 with those from 1988 to 1989. The 21 strains from 1988 to 1989 had all previously been characterized by MLEE as belonging to subgroup III and were serotyped as A:4/21:P1.20,9 (19). Antibiotic testing of these 21 strains and 3 strains from 2000 to 2001 revealed a susceptibility pattern identical to that of the strains from 2002 to 2003. To further study the phenotypic and genetic variation of the available strains from Ethiopia, we characterized all 64 strains from 1988 to 2003 for antigenic variation in their Opa proteins, their NadA proteins, and their LOS (Table 3).

Dot blotting showed that in contrast to the similarity in capsule serogroup, PorA, and PorB, the strains were less homogenous in their reactions with MAbs towards LOS and Opa proteins (Table 3). Except for OpaA and L3,7,9 reactions, there were no significant differences between old and recent strains, although a larger number of strains should be tested for conclusive results. OpaC was more frequently seen in recent strains than in old strains (P = 0.03). Opa5i was exclusively found in the old strains. Reaction with a MAb specific for the NadA protein was higher in recent strains than in old strains, although the difference was not significant (Table 3) (P = 0.246).

LOS types L11 and L10 were predominant among both the old and recent strains, with most of the strains showing an L11 reaction (Table 3). The L3,7,9 reaction was only seen in four of the old strains, and the difference was significant (P = 0.03). While only 1 of the old strains showed multiple LOS MAb reactions (strain Eth 9), 7 of the 43 recent strains did (Table 3). One strain from 1988 to 1989 (Eth 38) did not react with any of the anti-LOS MAbs used.

Overall, OM extracts of the strains were homogenous as judged by gel electrophoresis (Fig. 2 and 3). However, as in the dot blot analysis, differences were observed in expression levels of PorA and NadA and the band pattern of Opa proteins and LOS. Only three (8%) recent strains showed reduced amounts of the PorA protein in their OM extracts, and these strains also reacted weakly with the anti-PorA MAb in dot blotting. In general, the expression of PorB was higher than that of PorA. Some strains expressed high amounts of a protein of ~270 kDa; which was identified as the NadA protein by immunoblot. The expression correlated well with the intensity of the reaction with a NadA-specific MAb in the dot blot. Some variation in migration was seen for a band at ~70 kDa, which was probably the FetA protein, as seen by immunoblot analysis. Tdh1, Omp85, PorB, RmpM, and NspA proteins were detected by immunoblotting.
zymes showed that all 43 strains from 2000 to 2003 had the PCR product with ApoI, HincII, and SspI restriction en-

tability in subgroup III strains (2, 57), the tbpB gene was chosen to be characterized for genetic variation. A tbpB PCR product of 2.1 kb, typical of the isotype II tbpB gene, was present in all strains. Restriction fragment length polymorphism analyses of the PCR product with ApoI, HincII, and SspI restriction en-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Geographic origin, yr</th>
<th>ST</th>
<th>Major LOS</th>
<th>NadA Gene (TAAA),a</th>
<th>lgt-1 allele</th>
<th>lgt-2 allele</th>
<th>lgt-3 allele</th>
<th>tbpB allele</th>
<th>fetA allele</th>
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<tr>
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<td>-</td>
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<td>+</td>
<td>9</td>
<td>-</td>
<td>-</td>
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<td>7</td>
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a, +, clearly positive reaction with MAb; −, no reaction or no MAb.
b, n, number of TAAA repeat motifs.
c, PCR results with number of guanine (G) residues in the homopolymeric tract. The gene is predicted to be switched “on” if the tract length is 5 or 11 Gs, while the gene is predicted to be “off” if the tract length is 7 or 10 Gs (5).
d, A/Z, Addis Ababa or Zewai; G, North Gondar Zone; S, Sidama and Gedio Zones; USA, United States.
e, Control strains.
f, ND, allele not determined.

and were present in similar amounts in all OM extracts. The amount and migration pattern of Opa proteins were highly variable. In some strains, two unidentified bands were present at approximately 20 to 25 kDa (unknown) (Fig. 2).

Visualization of LOS expression by silver staining revealed one of two bands (Fig. 3) in most strains: the upper band was confirmed as either L10 or L3,7 and the lower band was confirmed as either L11 or L8 by immunoblotting. OM extracts that reacted with both L10 and L11 MAbs in the dot blot had two bands but in different amounts. Strains reacting with the L3,7,9 MAB in the dot blot showed one major band, as confirmed by immunoblotting, where the MAbs MN15A8-1 and 9-1-L3,7,9 showed similar reactions. Strain Eth 38, which did not react with any of the tested anti-LOS MAbs, showed two bands on the TSDS-PAGE gel (Fig. 3). The LOS type in this strain could be L13 on the basis of electrophoretic migration by comparison with an L13 strain from Sudan (Fig. 3, lanes 14 and 17) (42), but it could also be of an immunotype for which MAbs were not available to us.

Genotypic comparison of the strains from 2002 to 2003 with those from 1988 to 1989. Sequencing of the pgm locus from the strains isolated from 1988 to 1989 revealed allele 3, and thus, they were assigned to ST-5. The three strains from 2000 were also genotyped by MLST and were assigned to ST-7.

Based on results from previous studies on microheterogeneity in subgroup III strains (2, 57), the tbpB gene was chosen to be characterized for genetic variation. A tbpB PCR product of 2.1 kb, typical of the isotype II tbpB gene, was present in all strains. Restriction fragment length polymorphism analyses of the PCR product with ApoI, HincII, and SspI restriction enzymes showed that all 43 strains from 2000 to 2003 had the same restriction fragment band pattern, compatible with that expected for the tbpB55 allele. Of the 21 strains from 1988 to 1989, 20 presented with a restriction enzyme fragment band pattern similar to that of the tbpB1 control strain (Z1054), and one single strain (Eth 12) presented with a different band pattern comparable to that expected for allele 38 (Table 4 and Fig. 4). Sequencing of the tbpB gene in strains showing different tbpB restriction patterns confirmed the presence of tbpB1 and tbpB55, while the tbpB sequence of strain Eth 12 showed
Fourteen strains representing different LOS types and different expression levels of PorA and NadA were selected for a more thorough comparison of genetic variation. PCR and sequencing of the fetA gene in these 14 strains showed that all encoded epitope F3-1, irrespective of whether they were ST-5 or ST-7 strains. Alignment of the fetA sequences, however, showed that while the eight ST-7 strains were identical to the fetA07 allele in the region sequenced, all six ST-5 strains had the fetA11 allele (Table 4).

PCR of the nadA gene showed that all 14 strains (Table 4) possessed a nadA gene with a similar size. Sequencing of the nadA gene from one strain (Eth 35) showed it to be of allele 3. Sequencing of the promoter area of the nadA gene revealed 6 to 13 copies of the TAAA repeat motif (Table 4). The number of repeats in the 14 strains correlated with the expression of NadA seen with MAb 1079-B6 in the dot blot: there was low or no reaction with 6, 9, and 12 TAAA repeats; moderate reaction with 11 repeats; and strong reaction with 8 and 13 repeat motifs.

PCR of the nine lgt genes showed two patterns in the 14 selected strains: pattern 1, with the presence of lgtABHFG, and pattern 2, with the presence of lgtAHFG (Table 4). On the basis of the organization of their lgt genes, the A-4:21:Pl.20.9 strains appeared to belong to LOS genotypes 3 (VII-I-I) and 8 (VIII-I-I) (56). The presence or absence of lgtB was therefore analyzed for all strains, and the results are given in Table 3. There was a statistically significant association between the L11 immunotype and the lack of lgtB (P < 0.0001). Also, there was a significantly higher proportion of lgtB-positive strains originating from the SNNPR (18/27) than from Gondar (2/13) (P = 0.006), while the proportion was similar in old (10/21) and recent (21/43) strains. Seven of the eight L11 strains positive by lgtB PCR that were not included among the subset of 14 isolates in Table 4 were also analyzed for the presence of the other eight lgt genes. All genes showed pattern 1. Curiously, the ST-5 control strain, Z1054, isolated in Finland in the 1970s showed no presence of the lgtG gene in PCR, thus differing from the ST-5 Ethiopian strains (Table 4). Our PCR results for the reference strains were as previously reported (56), except for a positive PCR result for the lgtB gene in strain 126E (Table 4). Sequencing of this PCR product revealed a sequence that was not similar to any Neisseria lgt sequence in GenBank but that had 97% identity to gene NMA0505, encoding a putative ABC transport ATP-binding subunit.

Sequencing of the lgtB gene from three Ethiopian strains, one ST-7 (Eth 02) and two ST-5 (Eth 9 and Eth 12) strains, showed that the gene fragment was identical to that of lgtB in strain Z2491 (56). We also explored the sequence variation in the genes lgtA and lgtG using two ST-5 (Eth 9 and Eth 12) and two ST-7 (Mk 686/02 and Mk 804/03) strains. For lgtA, the ST-5 strains were identical in the sequenced fragment to allele 11, while the ST-7 strains were identical to each other and showed high similarity with lgtA17 (502/523 nucleotides). This pattern was further confirmed for the 10 other Ethiopian strains listed in Table 4. All new strains possessed a homopolymeric tract of five guanine residues enabling a functional lgtA gene product, while old strains showed diverse tract lengths (Table 4). For the lgtG gene, the four sequences were identical and showed the highest sequence similarity (547/548 nucleotides identical) to the lgtG9 and lgtG10 alleles. Larger fragments must be sequenced in order to definitively identify the specific lgtG alleles these strains harbor. Different lengths of the homopolymeric cytosine tract were found; however, both ST-5 strains had a tract with 9 residues, and both ST-7 strains had a tract with 10 residues. With these lengths, the gene is predicted to be switched off (5). For the lgtH gene, we found that seven out of eight ST-7 strains and four out of six ST-5 strains harbored the lgtH3 allele. The remaining ST-7 strain was identical except for a single point mutation to lgtH3 (Mk 499/03), while the remaining two ST-5 strains were either identical (Eth 18) or identical except for a single point mutation (Eth 9) to the lgtH gene of strain Z2491.

**DISCUSSION**

**Clinical data.** Of the 95 patients enrolled in the study, 71 had confirmed meningococcal disease by culture or PCR of the CSF. The CFRs found in this study were within the ranges of those reported overall in Ethiopia from 2002 to 2003 (Table 1). Among 132 children ≤14 years of age in Gondar from 1990 to 1994, the CFR for bacterial meningitis was 28%, while the N. meningitidis-specific CFR was 16% (15). CFRs for epidemic meningococcal disease in the meningitis belt range from 3 to 30% (6, 17, 26). They are probably underestimated due to the fact that septicemic patients might die before reaching the health facility (17, 25). The low N. meningitidis-specific CFR observed in our study (4.2%), with few of the patients confirmed with meningococcal disease presenting with ecchymoses or petechiae (Table 2), is most likely due to our inclusion criteria, which focused on the clinical signs of meningitis. The CFR among the 24 cases not confirmed as meningococcal disease was significantly higher. This fits with the observation that meningitis caused by, e.g., S. pneumoniae or H. influenzae, the other major agents of meningitis in Africa, is associated with high CFRs (39). Other microbes might also have been responsible for these unconfirmed cases.

**Recovery of meningococci.** In this study, survival of meningococcal strains in CSF inoculated in T-I medium lasted for up to 67 days. Recovery of meningococci from the T-I medium seemed to depend more on factors other than the duration of transportation alone. The porA-specific PCR increased the percentage of patients confirmed as being positive for N. meningitidis from 42% to 75%, showing the benefit of PCR in ascertaining the burden of meningococcal meningitis. Although such a benefit is evident from numerous studies in the meningitis belt (21, 38, 45), even the most basic reagents and equipment remain scarce in hospital laboratories in Ethiopia; only a few reference laboratories in the meningitis belt can afford the relatively costly PCR method for routine testing. Long-term general-capacity building of regional microbiology laboratories and national production of transport medium, e.g., modified T-I medium (M. J. Hughes, M. A. Chang, G. W. Ajello, S. Diarra, F. Bougoudogo, S. E. Schmink, G. A. Barnett, P. L. Raghunathan, T. Popovic, and L. W. Mayer, Abstr. 14th Int. Pathogenic Neisseria Conf., abstr. 94, 2004), might be a more fruitful first step for improving diagnostics.

**Characterization of recent strains.** Only genetically and antigenically very homogenous N. meningitidis strains of sero-
group A, serotype 4/21, serosubtype P1.20.9, and ST-7 caused the meningitis epidemic in Ethiopia in 2002 and 2003. All strains proved susceptible to all tested antibiotics except sulphonamide, which is in line with other studies in the meningitis belt (12, 14, 19, 22, 38, 42). Resistance to sulphonamide in meningococcal isolates from Ethiopia was seen already in 1970 (55). Resistance to penicillin G and to chloramphenicol (43), which has emerged in other parts of the world in the last two decades, has not yet been documented in the meningitis belt (48). Although some Ethiopian meningococcal strains have been reported to be resistant to chloramphenicol (33), these isolates were not available for confirmation and further characterization.

### Comparison of strains from 2000 to 2003 to those from 1988 to 1989

The strains from the epidemic of 1988 to 1989 were all ST-5, while those collected from 2000 to 2003 were ST-7. ST-7 meningococci were first detected on the African continent in Algeria in 1995 (34, 57). Thus, the replacement of ST-5 with ST-7 in Ethiopia must have happened between 1995 and 2000. In a neighboring country, Sudan, ST-5 meningococci caused the epidemic of 1988 to 1989 (22), while the large epidemic of 1999, with over 33,000 cases, was caused by ST-7 (34). ST-7 has not yet caused epidemics of similar magnitude in Ethiopia. In recent years, additional STs within the ST-5 clonal complex have appeared in Africa: ST-580 in Burkina Faso in 1997 (34), ST-2144 in Sudan and ST-581 in Senegal in 1999, and ST-203 in Gambia and ST-2207 in Burundi in 2002. Also, strains of ST-2859 were reported in Niger and Burkina Faso in two consecutive seasons (2003 and 2004) (http://pubmlst.org/neisseria) (35, 36). The presence of multiple STs in the ST-5 complex might result from the selection of variants in response to changes in the immunity of the populations. This may be a challenge, and close surveillance is required to enable appropriate preventive measures to be initiated in time.

The significance of the phenotypic differences between the recent ST-5 complex strains and the older ones is difficult to judge, as phenotypic data of this kind only provide a “snapshot” of antigen expression under the given in vitro conditions. Still, the results of our dot blot analyses of the Ethiopian isolates from 1988 to 1989 were consistent with those of subgroup III strains from Sudan in 1988 (42) and cases related to the Mecca outbreak of 1987 to 1988 (1); OpC, OpA5a, OpA5f, and OpA5i, for example, were expressed in similar proportions (Table 3). In recent strains, however, the OpA reaction pattern showed the presence of OpA5a, OpA5f, and OpA5h. Some strains did not react with our panel of Opa MAbs, suggesting that new variants might have arisen. A relatively limited number of immunotypes was observed. Immunotypes L10 and L11 predominated, as previously observed in the “Mecca-related” and the Sudanese subgroup III strains (1, 42).

### Microvariation in subgroup III strains from Ethiopia analyzed in this study

<table>
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<tr>
<th>Yr</th>
<th>Origin</th>
<th>ST</th>
<th>Allele variant (no. of isolates with allele/no. of isolates analyzed)</th>
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<tr>
<td>1988–1989</td>
<td>Addis Ababa or Zewai</td>
<td>5</td>
<td>3 (21/21)</td>
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<td>2000–2001</td>
<td>Oromiya and Amhara regions</td>
<td>7</td>
<td>19 (3/3)</td>
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<tr>
<td>2002–2003</td>
<td>Gondar and SNNPR</td>
<td>7</td>
<td>19 (40/40)</td>
</tr>
</tbody>
</table>

* Data are from isolates listed in Table 4.
* Data are from isolates Eth 9, Eth 12, and Mk 686/02.

The protein patterns of the strains, as observed by SDS-PAGE, were very similar, with all strains showing the major proteins TdH, Omp85, PorB, RmpM, Opa, and NspA (35). However, the expression of PorA, OpC, and NadA and the repertoire of Opa proteins were variable. The level of NadA expression correlated with the number of TAAA repeats in the promoter region of the gene, as reported previously by Martin et al. (32). The significance of this variation for the ability of NadA to mediate adhesion and to induce an immune response remains unclear (32).

We further characterized allelic variation in the genes encoding two proteins with a high degree of variation, TbpB and FetA. TbpB is a surface-exposed protein thought to be important for immunity towards meningococci (29). In line with previous studies of subgroup III strains (57), we found that all the ST-7 strains harbored allele 55 (genocloud 8), while most ST-5 strains harbored allele 1 (genocloud 5). One ST-5 strain (Eth 12) recovered from the epidemic of 1988 to 1989, however, harbored a new allele, which could have been imported anew by DNA transformation from other neisseriae (29). The presence of the opaB94 allele in Eth 12 (Table 5) confirmed that the strain belonged to genocloud 5 (2, 57) and that the tbpB allele was imported independently in ST-5 in Ethiopia.

FetA is a hypervariable and phase-variable iron-regulated outer membrane protein to which antibodies are induced following disease or vaccination (47). Most of the variation is found in loop 7 of the proposed FetA topology model and allows for the designation of FetA epitope variants (Fig. 5) (47, 50). The six ST-5 and the eight ST-7 strains all had FetA epitope F3-1. However, the ST-5 strains had the fetA11 allele, while the ST-7 strains had the fetA07 allele. This is in agreement with the analysis of 10 subgroup III strains (47, 49), where fetA alleles 5, 7, 11, 54, and 55 were found among the ST-5 strains and the only ST-7 strain analyzed harbored allele 7. Thus, fet4 might be considered another important indicator of microvariation in subgroup III strains. While fetA11 and fetA07 are encoding the same peptide variant, F3-1, they differ from one another in 32 amino acids outside the defined variable region. Most of these differences are located in areas likely to be surface exposed, more specifically, in loops 5, 6, 8, 10, 11, 12, and 13. FetA loops other than the defined main epitope could possess the ability to induce a functional immune response (50); one could speculate that this might be relevant for immune selection, and it should be a subject of

---

**Table 5. Microvariation in subgroup III strains from Ethiopia analyzed in this study**

<table>
<thead>
<tr>
<th>Yr</th>
<th>Origin</th>
<th>ST</th>
<th>Allele variant (no. of isolates with allele/no. of isolates analyzed)</th>
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<tr>
<td>1988–1989</td>
<td>Addis Ababa or Zewai</td>
<td>5</td>
<td>3 (21/21)</td>
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<td>2002–2003</td>
<td>Gondar and SNNPR</td>
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</table>
further investigation. The full extent of fetA variation needs to be analyzed in a larger collection of ST-5 complex/subgroup III meningococci from multiple countries and time periods.

LOS genotyping of the Ethiopian strains revealed a difference in the occurrence of lgtB. The difference was significantly associated with the geographic origin in the recent strains (P = 0.006); it was not associated with the time of collection (Table 3). lgtB was lacking in strains expressing the L11 LOS type alone (Table 3), implying that lgtB is involved in the biosynthesis of the non-L11 immunotypes. A lack of lgtB is known to result in the loss of the terminal galactose in the lacto-N-neotetraose structure in the oligosaccharide α-chain (16), whereby a strain cannot synthesize L3,7,9 (5). Deletion of the lgtB gene could occur through recombination between repeated DNA fragments at the flanking regions (56), a mechanism that could facilitate escape from the host response. Considering the LOS types (L3,7,9, L8, L10, L11, and L13) expressed in our strains, the observed LOS antigenic variation could also have been caused by the on/off switching of lgtA and lgtG mediated by variable homopolymeric repeat tracts (16), by allelic variation of the lgt genes, or by creation of new mosaic lgtH alleles due to intragenic recombination (56). In addition, the presence or absence of lpt-3, which is required for the synthesis of LOS types L1, L3, L7, and L8 (30), could have contributed to the observed variation. LOS genotyping by simply mapping the presence of the nine lgt genes (56) did not enable the epidemiologically relevant differentiation of the ST-5 complex strains from Ethiopia. However, our study identified different lgtA alleles in ST-5 and ST-7 strains, indicating that LOS biosynthesis-associated genes among ST-5 complex strains could be useful as additional markers of microevolution. However, the impact of allelic variation in lgtA on LOS antigenic structure or virulence is not clear.

Analysis of selected genes in subgroup III meningococci enabled the subdivision of the clonal complex into nine genoclouds (57). Our study showed that microheterogeneity in subgroup III strains occurred in additional genes, such as fetA and lgtA. The comparative proteomic approach, which recently was validated by tbpB analysis of strains from different genoclouds, has identified multiple proteins, which could be useful for resolving fine phylogenetic relationships of the strains (4). The evidence of further differences in antigenic structures among clones of the ST-5 complex, e.g., in TbpB and FetA, might explain the replacement of ST-5 by ST-7 in the African meningitis belt. Serological studies should be performed to test this hypothesis. Alternative hypotheses that could explain this replacement include short-lived or nonprotective immune response following disease, coinfections, or environmental changes.

The meningitis epidemics in northern and southern Ethiopia in 2002 and 2003 were caused by serogroup A. N. meningitidis strains of ST-7, which were antigenically and genetically very homogeneous. In this epidemiological situation, polysaccharide conjugate vaccines (23), as well as outer membrane protein-based vaccines (37), could provide long-lasting immunological protection. An affordable conjugate vaccine against serogroup A meningococcal disease will hopefully be available for countries in the meningitis belt within the next decade. Prior to the introduction of these vaccines, country-specific estimates of meningococcal disease burden and serogroup determination of representative disease isolates are required to evaluate their potential impact, as these are the major factors for determining effectiveness, besides vaccine efficacy. Although only serogroup A meningococci were found in our study, serogroup W135 epidemics occurred in Burkina Faso in 2001 and 2002, and an outbreak of W135 meningococci was reported in a neighboring country, Sudan, in 2005 (http://www.who.int/csr/don). The Ethiopian health authorities should therefore ensure their laboratory-based surveillance network in order to detect potential meningococcal strain heterogeneity to be able to provide the appropriate vaccine in time.

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