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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] Wegene Tamire,[7] E. Arne Høiby,[1] Tsegaye Alebel,[8] Degu Berhanu,[2] Yarid Merid,[4] Morten Harboe,[2,9] and Dominique A. Caugant[1,10]*

Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway[1]; Armauer Hansen Research Institute, Addis Ababa, Ethiopia[2]; Liverpool School of Tropical Medicine, Liverpool, United Kingdom[3]; Southern Nations, Nationalities, and Peoples' Region Health Bureau, Awassa, Ethiopia[4]; Gondar College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia[5]; Department of Internal Medicine, Faculty of Medicine, Addis Ababa University, Addis Ababa, Ethiopia[6]; Sidamo Regional Hospital, Yirgalem, Ethiopia[7]; North Gondar Zone Health Bureau, Gondar, Ethiopia[8]; Institute of Immunology, University of Oslo and Rikshospitalet University Hospital, Oslo, Norway[9]; and Department of Oral Biology, University of Oslo, Oslo, Norway[10]

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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 confirmed as having *Neisseria meningitidis* either by culture ($n = 40$) or by *porA* PCR ($n = 31$) of their CSF. The overall case fatality rate (CFR) was 11.6%; the *N. meningitidis*-specific CFR was 4.2%. All 40 strains were fully susceptible to all antibiotics tested except 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.

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/don). Molecular epidemiological studies have shown that serogroup A strains are genotypically diverse, but specific complexes of related hypervirulent clones are responsible for a major part of the cases in the meningitis belt (8). Most recent epidemics have been caused by a clonal group introduced to the meningitis belt from Mecca, Saudi Arabia, in 1987; it was first identified using multilocus enzyme electrophoresis (MLEE) and was called subgroup III (8, 57). Serogroup A meningococci of subgroup III are very homogenous: basically, all of the meningococci express a serosubtype P1.20,9 PorA and a serotype 4/21 PorB (8). In spite of the capability of rapid genetic change through genomic rearrangements or uptake of foreign 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).

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

* Corresponding author. Mailing address: Department of Bacteriology and Immunology, Division of Infectious Disease Control, Norwegian Institute of Public Health, P.O. Box 4404, Nydalen, NO-0403 Oslo, Norway. Phone: 47 22 04 23 11. Fax: 47 22 04 25 18. E-mail: dominique.caugant@fhi.no.

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

Yr	Whole country[a]			North Gondar Zone, Amhara region[b]				Sidama and Gedio Zones, SNNPR[c]			
	No. of cases	No. of deaths	CFR[d] (%)	No. of cases	No. of deaths	CFR (%)	No. vacc[e] (%)	No. of cases	No. of deaths	CFR (%)	No. vacc (%)
2000	855	19	2.2	0	0	0		533	15	2.8	226,395 (18)
2001	6,266	311	5.0	384	26	7	473,300 (41)	1,345	52	3.9	825,413 (58)
2002	2,329	118	5.1	1,235	128	10.4	214,408 (26)	346	0	0	377,794 (98)
2003	3,540	166	4.7	0	0	0		1,010	44	4.4	279,561 (26)

[a] Data source, http://www.who.int/csr/don.
[b] 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.
[c] Data sources, SNNPR Regional Health Bureau, Central Statistical Authority, Addis Ababa, Ethiopia, and ORC Macro, Calverton, MD.
[d] CFR is defined as the number of deaths attributed to meningitis per number of patients with meningitis.
[e] 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 objectives of this study were to collect meningococcal strains from Ethiopia from 2002 to 2003, to characterize 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.

(Part of this work was presented at the 14th International Pathogenic *Neisseria* Conference in Milwaukee, WI, September 2004.)

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).

MATERIALS AND METHODS

Study area and population. The study was conducted in the Sidama and Gedio Zones in the SNNPR and in the North Gondar Zone in the Amhara region of Ethiopia between April 2002 and December 2003. The Sidama and Gedio Zones are located in the lowlands of southern Ethiopia, within an area with a tropical climate. Most of the patients in the SNNPR were admitted to Yirgalem and Dilla hospitals and the Bushulo Major Health Center, all of which are located within 80 km south of Awassa (Fig. 1) and predominantly serve a rural community (approximately 3 million people). The North Gondar Zone is located in the highlands of northern Ethiopia and is part of the traditional meningitis belt (26). Most of the patients in the North Gondar Zone were admitted to Gondar University Hospital, which is located 750 km north of Addis Ababa and serves the population of the town of Gondar (Fig. 1) and the surrounding zones (approximately 4.5 million people). Patients were also admitted to Metema hospital, 160 km west of Gondar, and to health centers in remote villages.

Layout of the study and clinical diagnosis. This prospective study included patients from the two meningitis seasons of 2001 to 2002 and 2002 to 2003. Suspected meningitis cases presenting at the study sites were included on the basis of the following criteria: (i) being clinically diagnosed with meningitis (53), (ii) having turbid cerebrospinal fluid (CSF), and (iii) being aged 6 months and older. In each region, patients fulfilling the inclusion criteria were recruited consecutively in the first year until at least eight patients were included in each of the following desired age groups: infants (6 months to up to 2 years of age), young children (2 to <6 years of age), older children and teenagers (6 to <15 years of age), young adults (15 to <20 years of age), and adults (20 years of age and above) for the purpose of serological analyzes. During the second season (2002 to 2003), recruitment focused on including children younger than 2 years of age, as too few were recruited during the first season. Consequently, the age distribution in this study does not necessarily reflect the true distribution among meningitis patients in Ethiopia.

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 hospitals. Lumbar puncture for CSF sampling was carried out as a 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 (vancomycin [3.0 μg/ml], colistin methane sulfonate [7.5 μg/ml], nystatin [12.5 U/ml], and trimethoprim [5.0 μg/ml]) (Oxoid Ltd., Basingstoke, 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.

In addition, 21 strains collected in Addis Ababa and the town of Zewai (170 km south of Addis Ababa) in Ethiopia in 1988 and 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 126E, M986, 44/76, Z1054 (47, 56, 57) (see Table 4), and 188/87 were obtained from the same collection for use as control strains in PCR analyses. *Streptococcus pneumoniae* ATCC 49619 and *Escherichia coli* ATCC 25922 were used for quality control purposes in antibiotic sensitivity testing as described previously (10).

Protein and LOS characterization. Serotypes, serosubtypes, immunotypes, Opa protein repertoire, and OpcA and NadA protein expression were determined by dot blot with whole-cell preparations, as described previously (52). We used the monoclonal antibodies (MAbs) (with the designations in parentheses): P1.7 (MN14C11-6), P1.9 (MN5A10F), P1.20 (V502), P3.4 (15-1-P4), P3.21 (14-1-P21), L1 (223 D-8), L3,7 (MN15A8-1 and 9-1-L3,7,9), L8 (2-1-L8), L10 (14-1-L10), L11 (4C4), OpcA (279/5c), Opa5a (W320/15), Opa5f (AB419), Opa5h (U205), Opa5i (T116), NadA (1079-B6), and NspA (236 B-2) (references for MAbs and polyclonal sera used are given in reference 37). OM extracts were prepared using the LiCl/LiAc method (13) and stored at −20°C. The total content of proteins was determined using the Bio-Rad DC protein assay (Bio-Rad Inc., Richmond, CA). OM extracts were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by applying approximately 3 μg protein from each OM extract onto 12% gels, followed by Coomassie brilliant blue staining. LOS bands in the OM preparations were separated and visualized by applying approximately 120 ng protein from each OM extract onto a 16.5% tricine-SDS-PAGE (TSDS-PAGE) gel, followed by silver staining (28). To identify the positions of major antigens in the OM extracts, immunoblotting was carried out as described previously (51) by using the MAbs listed above or polyclonal antisera against the TdfH or Omp85 proteins.

Antibiotic sensitivity. All 64 *N. meningitidis* strains from Ethiopia (21 from 1988 to 1989, 3 from 2000 to 2001, and 40 from 2002 to 2003) were tested by the Etest method (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions, with chocolate Mueller-Hinton agar (20). Susceptibility to the following drugs was tested: penicillin G, ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, rifampin, and sulfamethoxazole. CLSI susceptibility criteria were applied (10).

MLST. MLST was performed as described previously (31). Briefly, fragments of each of the seven genes *abcZ*, *adk*, *aroE*, *gdh*, *pdhC*, *pgm*, and *fumC* were amplified by PCR on a boiled bacterial cell suspension. Sequencing of the PCR products was performed on both strands using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing reactions were run on an ABI Prism 377 instrument (Applied Biosystems) using 5% Long Ranger gels (FMC Bioproducts, Rockland, ME). Alleles and STs were assigned, as described previously (31), by querying the *Neisseria* MLST database (http://pubmlst.org/neisseria).

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 retested 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.

Genetic characterization of the strains. The *nadA* gene was amplified using primers and PCR conditions as described previously (11). Similarly, the nine glycosyltransferase genes *lgtA* to *lgtH* and *lgtZ* were amplified as described previously (56). Selected PCR products of the *nadA* promoter region, *lgtA*, *lgtB*, *lgtH*, and *lgtG* were sequenced as described above. Additional primers were used for sequencing of *lgtA* (lgtA_M2 [5′-AGC GTT TCC AAG AAC AGG AC-3′] and P6 [56]) and *lgtH* (lgtH_M1 [5′-CGC CGT ATT TGA AGA TGA TG-3′] and P26 [56]). The *fetA* 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* FetA variable region database (http://neisseria.org/nm/typing/feta). Analyses were based on the alignment of the ~410-nucleotide-long sequences with the 81 currently available *fetA* sequences on this website. The *tbpB* gene was amplified using primers OTG6687 and OTG6689 (27). Following purification of the *tbpB* PCR product with the QIAquick purification kit (QIAGEN), macrorestriction fragments were obtained by digestion of the purified *tbpB* product using the restriction endonucleases ApoI and SspI (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 *tbpB* PCR products from a control strain, Z1054 (*tbpB* allele 1). The band patterns with restriction enzymes ApoI, HincII, and SspI of *tbpB* alleles 1, 38, 39, 55, and 59, which are the dominant alleles in subgroup III meningococci (57), were predicted using the software program NEBcutter (version 2.0; New England Biolabs Inc. [http://tools.neb.com/NEBcutter2]). The *tbpB* gene of strains with different restriction fragment patterns was sequenced using primers 3′Met2 and O1641 (29) for old strains and T55_F (5′-TGT TGA GTG CTT GTC TGG GC-3′) and T55_R (5′-TCC CCG GAA AAA GCA CTA TA-3′) for recent strains. The *opaB* gene was amplified using primers O464 and O3510 (2) and sequenced using primers OpaB_p1 (5′-TAT CGG TGT GCG CGT C-3′) and OpaB_p2 (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 chi-square test or 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 nonparametric 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 *tbpB*, *lgtA*, and *lgtH* (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

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

Characteristic	Value for group[a]	
	All patients	*N. meningitidis* meningitis[b]
Demographic		
Residence		
North Gondar Zone	23/95 (24)	19/71 (27)
Sidama and Gedio Zones	72/95 (76)	52/71 (73)
Yr		
2002	51/95 (54)	38/71 (54)
2003	44/95 (46)	33/71 (46)
Age (yr)		
0.5–<2	7/93 (8)	3/70 (4)
2–<6	12/93 (13)	10/70 (14)
6–<15	29/93 (31)	25/70 (36)
15–<20	15/93 (16)	12/70 (17)
≥20	30/93 (32)	20/70 (29)
All[d]	16.0 (13.6–18.3), 14	15.0 (12.6–17.5), 14
Sex		
Male	54/93 (58)	38/70 (54)
Female	39/93 (42)	32/70 (46)
Preadmission history		
Antibiotics given before admission	10/90 (11)	6/67 (9)
Previously vaccinated with serogroup A polysaccharide vaccine	15/90 (17)	11/67 (16)
Days of illness from first symptoms to examination at hospital (n = 92; range, 0–9)[d]	2.9 (2.6–3.3), 3	2.9 (2.5–3.3), 2
Clinical and laboratory findings at hospital admission		
Nuchal rigidity	89/89 (100)	66/66 (100)
Back rigidity	34/58 (59)	23/41 (56)
Ecchymoses	7/71 (10)	5/56 (9)
Petechiae, ≥10	15/39 (38)	11/28 (39)
Seizures	12/91 (13)	8/68 (12)
Shock	4/90 (4)	2/67 (3)
Coma	14/91 (15)	7/68 (10)
End result[c]		
Death	11/95 (12)	3/71 (4)
Sequelae	3/95 (3)	2/71 (3)
Days of stay at hospital (n = 47; range, 0–15)[d]	6.5 (5.5–7.4), 7	6.3 (5.2–7.3), 7

[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% CIs), medians.

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

Antigen	No. (%) of positive reactions for time period[a]:		*lgtB* positive[b] (n = 64)
	2000–2003 (n = 43)	1988–1989 (n = 21)	
OpcA	36 (84)	10 (48)	
Opa type			
5a	7 (16)	4 (19)	
5f	13 (30)	4 (19)	
5h	3 (7)	0	
5i	0	2 (10)	
5af	5 (12)	6 (29)	
5ah	1 (2)	0	
5fh	3 (7)	0	
5ai	0	2 (10)	
NadA	10 (23)	8 (38)	
LOS types			
L3,7,9	0	3 (14)	3/3 (100)
L10	10 (23)	4 (19)	14/14 (100)
L11	26 (60)	12 (57)	4/38 (11)
L10, L11	6 (14)	0	6/6 (100)
L8, L11	1 (2)	0	1/1 (100)
L3,7,9, L8, L11	0	1 (5)	1/1 (100)
L13	0	1 (5)	1/1 (100)

[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.

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 data, 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).

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).

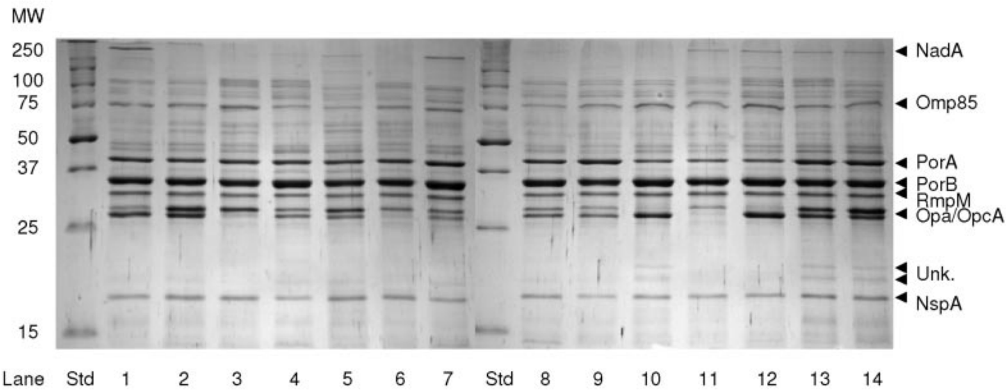

FIG. 2. Coomassie brilliant blue-stained 12% SDS-PAGE gels with OM extracts from *N. meningitidis* isolates listed in Table 4. Lane 1, Mk 499/03; lane 2, Mk 502/03; lane 3, Mk 804/03; lane 4, Mk 365/02; lane 5, Mk 686/02; lane 6, Mk 689/02; lane 7, Mk 691/02; lane 8, Mk 802/02; lane 9, Eth 2; lane 10, Eth 9; lane 11, Eth 12; lane 12, Eth 18; lane 13, Eth 35; lane 14, Eth 38. Unk.; unknown protein bands; Std, standard; MW, molecular weight (in thousands).

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 $\geq$ 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 opacity 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 OpcA 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. OpcA 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. TdfH, Omp85, PorB, RmpM, and NspA proteins were detected by immunoblotting

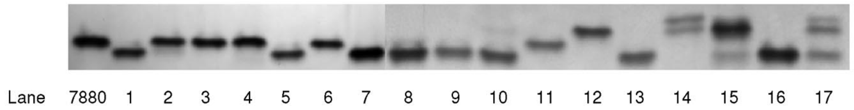

FIG. 3. Silver-stained LOS bands of OM extracts separated in 16% TSDS-PAGE gels. 7880, L10 prototype strain (24). Lanes 1 to 14 contain OM extracts from same strains shown in Fig. 2. Lane 15, strain 44/76 (L3,7,9); lane 16, strain N 144/95 (L8); lane 17, strain Sudan 433/88 (L13) (42).

TABLE 4. Characterization of 14 serogroup A subgroup III-1 meningococcal strains collected in Ethiopia from 1988 to 1989 and 2002 to 2003

Strain	Geographic origin, yr	ST	Major LOS	NadA			*lgt-1*							*lgt-2*	*lgt-3*	*tbpB* allele	*fetA* allele
				MAb[a]	Gene	$(TAAA)_n$[b]	*lgtA*[c]	*lgtB*	*lgtC*	*lgtD*	*lgtE*	*lgtZ*	*lgtH*	*lgtF*	*lgtG*		
Mk 499/03	S, 2003	7	L11	+	+	13	+ 5	−	−	−	−	−	+	+	+	55	7
Mk 502/03	S, 2003	7	L10, L11	−	+	9	+ 5	+	−	−	−	−	+	+	+	55	7
Mk 804/03	S, 2003	7	L10	−	+	9	+ 5	+	−	−	−	−	+	+	+	55	7
Mk 365/02	G, 2002	7	L10, L11	−	+	12	+ 5	+	−	−	−	−	+	+	+	55	7
Mk 686/02	G,[d] 2002	7	L11	+	+	11	+ 5	−	−	−	−	−	+	+	+	55	7
Mk 689/02	S,[d] 2002	7	L10	−	+	12	+ 5	+	−	−	−	−	+	+	+	55	7
Mk 691/02	G, 2002	7	L11	+	+	11	+ 5	−	−	−	−	−	+	+	+	55	7
Mk 802/02	S, 2002	7	L10, L11	−	+	6	+ 5	+	−	−	−	−	+	+	+	55	7
Eth 2	A/Z,[d] 1989	5	L11	−	+	9	+ 10	−	−	−	−	−	+	+	+	1	11
Eth 9	A/Z, 1989	5	L8, L3,7,9, L11	−	+	9	+ 7	+	−	−	−	−	+	+	+	1	11
Eth 12	A/Z, 1989	5	L10	+	+	8	+ 7	+	−	−	−	−	+	+	+	ET1	11
Eth 18	A/Z, 1989	5	L3,7,9	+	+	8	+ 11	+	−	−	−	−	+	+	+	1	11
Eth 35	A/Z, 1989	5	L11	+	+	8	+ 10	−	−	−	−	−	+	+	+	1	11
Eth 38	A/Z, 1989	5	"L13"	+	+	8	+ 11	+	−	−	−	−	+	+	+	1	11
126E[e]	Germany, 1964		L1		−		−	−	+	+	+	+	−	+	−	ND[f]	ND
M986[e]	USA	11	L3,7	−	+	12	+	+	−	−	−	−	+	+	+	ND	ND
44/76[e]	Norway, 1976	32	L8, L3,7,9, L11		−		+	+	−	−	+	−	−	+	+	ND	1
Z1054[e]	Finland	5	L11	−	+	9	+	+	−	−	−	−	+	+	−	1	7

[a] +, clearly positive reaction with MAb; −, unclear or no reaction with 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

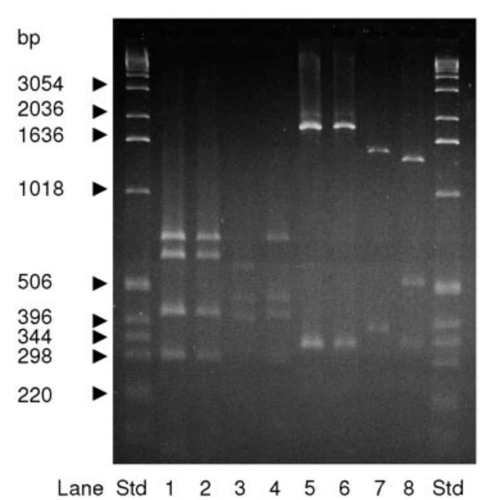

FIG. 4. Typical patterns of *tbpB* alleles previously found in subgroup III strains digested with the restriction enzyme ApoI (lanes 1 to 4) or SspI (lanes 5 to 8). Lanes 1 and 5, strain Z1054 (control allele 1); lanes 2 and 6, strain Eth 4 (allele 1); lanes 3 and 7, strain Eth 12 (new allele); lanes 4 and 8, strain Mk 502/03 (allele 55). Std, standard.

similarity to both *tbpB38* and *tbpB10* but probably represented a new allele.

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:P1.20,9 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 (E6-02) 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 $\leq$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-

TABLE 5. Microvariation in subgroup III strains from Ethiopia analyzed in this study[c]

Yr	Origin	ST	Allele variant (no. of isolates with allele/no. of isolates analyzed)				
			pgm	*tbpB*	*fetA*[a]	*lgtA*[a]	*opaB*[b]
2002–2003	Gondar and SNNPR	7	19 (40/40)	55 (40/40)	7 (8/8)	EL1 (8/8)	92 (1/1)
2000–2001	Oromiya and Amhara regions	7	19 (3/3)	55 (3/3)			
1988–1989	Addis Ababa or Zewai	5	3 (21/21)	1 (20/21) ET1 (1/21)	11 (6/6)	11 (6/6)	94 (2/2)

[a] Data are from isolates listed in Table 4.
[b] Data are from isolates Eth 9, Eth 12, and Mk 686/02.
[c] All characterized as A:4/21:P1.20,9 in dot blot.

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 sulfonamide, which is in line with other studies in the meningitis belt (12, 14, 19, 22, 38, 42). Resistance to sulfonamide 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 strains 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); OpcA, 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).

The protein patterns of the strains, as observed by SDS-PAGE, were very similar, with all strains showing the major proteins TdfH, Omp85, PorB, RmpM, Opa, and NspA (35). However, the expression of PorA, OpcA, 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, *fetA* 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

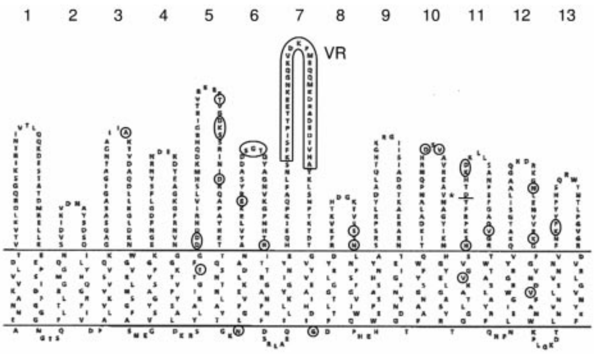

FIG. 5. Topology of FetA from strain H44/76 in the outer membrane (based on a figure by Pettersson et al. [40]), with the top part showing the surface-exposed loops and their numbers and with the VR marked with a box. Regions where the *fetA07* allele encodes different amino acids than the *fetA11* allele are marked with circles; in the VR, the *fetA07* and *fetA11* alleles encode similar amino acid sequences. *, in this position, *fetA07* encodes an insertion of two additional amino acids compared to *fetA11*.

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 enhance 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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