MAJOR ARTICLE

High Diversity of Group A Streptococcal *emm* Types among Healthy Schoolchildren in Ethiopia

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Background. Although the prevalence of rheumatic heart disease in Ethiopia is one of the highest in the world, the epidemiology of group A streptococci (GAS) in this country is little known. GAS typing is a hallmark of both the epidemiology and understanding of diseases caused by these organisms. We have therefore conducted this study to investigate the *emm* (M-protein gene) type distribution of GAS carriers among Ethiopian schoolchildren.

Methods. In the present study, we performed *emm* typing of 82 GAS isolates collected from the throats of healthy schoolchildren (6–14 years of age) residing in 3 different urban sites in Ethiopia: Addis Ababa, Gondar, and Dire Dawa.

Results. We report high diversity of GAS isolates recovered from healthy schoolchildren. Eighty-two isolates represented 43 different sequence types. Thirteen newly described subtypes were detected in this study. Of the *emm* types prevalent in the study communities, 46% were not included in the 26-valent GAS vaccine.

Conclusions. The high diversity of *emm* types encountered within 3 months of collection suggest that production of a vaccine candidate based on the M-protein amino termini appears to be impractical for this population. We suggest that investigations of other vaccine candidates, including the C5a peptidase, GAS carbohydrate, and fibronectin-binding proteins, as well as conserved M-protein region vaccines, should be intensified to address the needs of this population.

Group A streptococcus (*Streptococcus pyogenes;* GAS) is a bacterial pathogen that causes a wide spectrum of diseases, ranging from relatively benign conditions such as pharyngitis and pyoderma to more severe invasive diseases and also to the serious nonsuppurative sequelae: acute rheumatic fever and acute glomerulonephritis [1–4]. Children are the major reservoir of GAS and are the target population for pharyngitis as well as the suppurative and nonsuppurative complications. The only recognized natural host and, hence, reservoir for GAS is humans [5].

During the last century, there has been a steady decline in the incidence of serious streptococcal diseases in the developed world. However, in the 1980s, an increase in serious life-threatening infections due to GAS, such as necrotizing fasciitis, has been observed [6, 7]. In addition, simultaneous epidemiological observations strongly suggest an association with apparently more virulent GAS strains [8]. Nevertheless, the impact of serious group A streptococcal diseases, such as acute rheumatic fever, in many developing countries and even indigenous populations in industrialized countries is much greater [9–12].

Traditionally, GAS has been subclassified on the basis of serotype diversity of the M-protein, a major GAS virulence factor. The M-protein is a long coiled-coil protein projecting from the surface of the streptococcal cell wall. Amino acid variation in the amino-terminal portion of the protein serves as the basis for determining the M-type [13]. However, this method has several limitations, including the high cost and difficulty of keeping an entire set of specific M-typing antisera [14].

In recent years, DNA sequence–based methods for characterizing GAS strains have been introduced,

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including sequence analysis of *emm* gene–specific PCR products (*emm* typing) [15, 16]. This methodology has allowed the recognition of several previously unknown GAS types in different geographic areas, demonstrating the usefulness of *emm* typing for detecting genetic diversity among GAS isolates [15–17]. Many of these new *emm* types have been isolated from developing nations that have high levels of streptococcal infections [18–20].

In Ethiopia, rheumatic heart disease has an overall prevalence rate ranging from 4.6 [21] to 7.1 per 1000 children [22], which is among the highest rates in the world. Moreover, in up to 60% of admissions for cardiac problems in the children's hospital in Addis Ababa, the case had a rheumatogenic origin [23]. However, the epidemiology of GAS infection and its complications in this country is not well studied.

This study aimed at assessing the prevalent *emm* types circulating among healthy Ethiopian schoolchildren, the major reservoir and target population of the infections and complications. Study of the prevalence of healthy GAS carriers and the molecular epidemiology of the isolates may provide valuable information about the origin and spread of this infectious agent. Moreover, information regarding universal *emm* type distribution is useful for designing vaccines and developing vaccine strategies.

MATERIALS AND METHODS

Bacterial strains. A total of 82 GAS isolates were included in the present study. They were recovered from throats of 937 randomly selected healthy Ethiopian schoolchildren aged 6–14 years old between November 2004 and January 2005. Children with any signs or symptoms of upper respiratory tract infection were excluded. The isolates were collected from 7 schools in 3 major cities in the country: Addis Ababa ($n = 47$), Gondar ($n = 23$), and Dire-Dawa ($n = 12$). Gondar and Dire-Dawa are located 750 km north and 540 km southeast of Addis Ababa (capital of Ethiopia), respectively. Addis Ababa and Gondar have altitudes of 2400 m above sea level and 2200 m above sea level, respectively, in contrast with Dire-Dawa, which has an altitude of 1300 m above sea level. In Ethiopia, only 15% of the population reside in urban areas. The serogroup of the isolates was identified by Dry Spot streptococcal grouping kit (Oxoid). All isolates were stored in brain-heart infusion broth (Oxoid) with 15% glycerol at −70°C until processed.

DNA extraction, PCR, and primers. M-protein *(emm)* typing was performed according to the protocol described by the Centers for Disease Control and Prevention (CDC) with some modifications [24]. Enzymatic digestion of bacterial cells to prepare lysate was omitted, and DNA templates were instead prepared by boiling bacterial suspensions in distilled water [25]. A loopful of colonies was picked from an overnight growth on blood agar and suspended in 100 μL of distilled water. The suspension was then heated at 94°C for 2–3 min and used for PCR immediately or stored at −20°C until used. Primers 1 and 2 (5′-TATTCGCTTAGAAAATTAA-3′ and 5′-GCAAGTTCTTC-AGCTTGTTT-3′) were used as described by CDC [24]. PCR amplification was performed in a total volume of 50 μL of reaction mixture that contained 2 μL of the bacterial lysates, 5

Table 1. Distribution of *emm* types among Ethiopian group A streptococcal isolates from healthy schoolchildren.

emm Type	Geographic origin			Total no. of isolates
	Addis Ababa	Gondar	Dire-Dawa	
emm 1.0	2	0	0	2
emm 2	2	0	0	2
emm 3.19	11	1	0	12
emm 5.47[a]	2	0	0	2
emm 5.48[a]	0	4	0	4
emm 6.40[a]	1	0	0	1
emm 8	1	0	0	1
emm 12.0	0	0	1	1
emm 14.8[a]	0	1	0	1
emm 18.0	1	0	0	1
emm 28.0	1	1	0	2
emm 28.5[a]	1	0	0	1
emm 29.2	3	0	0	3
emm 29.5[a]	1	0	0	1
emm 30.2[a]	0	0	2	2
emm 38.1[a]	1	1	0	2
emm 39.2[a]	1	0	0	1
emm 42.5[a]	1	0	0	1
emm 43.7	0	1	0	1
emm 44/61.0	2	0	0	2
emm 53.4[a]	2	0	0	2
emm 56.0	1	0	0	1
emm 68.0	0	1	0	1
emm 68.5[a]	1	0	0	1
emm 74.0	1	0	1	2
emm 75.0	1	0	0	1
emm 80.0	0	0	1	1
emm 85.0	0	0	1	1
emm 86.2	0	1	0	1
emm 89.0	0	0	1	1
emm 90.3	1	0	0	1
emm 92.0	1	0	0	1
emm 93	0	0	1	1
emm 95.0	1	0	0	1
emm 102.4	0	1	0	1
emm 103.0	1	0	0	1
emm 106.2	1	1	0	2
emm 109.1	0	1	0	1
emm 119.3[a]	1	0	0	1
st62.0	0	4	0	4
st212.0	1	0	0	1
st430	0	1	1	2
st463.0	2	1	0	3
st854.1	0	1	0	1
st1731.1	1	0	1	2
st3757.0	0	1	2	3
st6735.0	0	1	0	1
Total no. of *emm* types/ no. of isolates	29/47	17/23	10/12	47/82

[a] Newly discovered subtype.

μL of 10× buffer PE GOLD, 5 μL of $MgCl_2$ (25 mmol/L), 2.5 μL each of primer 1 and 2 (10 pmol/μL), 1 μL of dNTP (1 mmol each), and 0.25 μL of AmpliTaq Gold. Amplification was done on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) using the cycle parameters as described by CDC: 94°C for 10 s; 10 cycles of 94°C for 15 s, 46.6°C for 30 s, and 72°C for 1 min 15 s; 20 cycles of 94°C for 15 s, 46.5°C for 30 s, and 72°C for 1 min 15s, with 10-s increment for each of the subsequent 19 cycles; followed by 72°C for 10 min; followed by 4°C storage. Amplification products were purified with JetQuick spin column technique (Germond) according to the manufacturer's instructions. PCR products were then run on 1% agarose gels to estimate the concentration of the products for the sequencing reaction.

Sequencing and BLAST analysis. About 4 μL of PCR product was sequenced using primer 1 (5′-TATTCGCTTAGAAAATTAA-3′) with the dye terminator mix, version 3.1 (Applied Biosystems). The sequencing products were purified by ethanol sodium acetate precipitation and subjected to automated sequence analysis on a 3100 model autosequencer (Applied Biosystems) as per the manufacturer's instructions. The cycling parameters were 96°C for 1 min for initial denaturation, followed by 25 cycles of 96°C for 10 s, 50 °C for 5 s, and 60°C for 4 min, and a holding temperature of 4°C. The Sequencher genetic software program, version 3.0, was used to edit the sequences. The *emm* gene sequence was subjected to homology search against CDC reference strains [26] as well as BLAST search analysis [27]. Pairwise comparison of the nucleotide homology for the first 150 bases of the hypervariable region was conducted. Strains that showed 100% homology with a reference strain were designated that particular parental *emm* subtype. Sequences that showed ⩾1 base pair discrepancies with reference sequences were sent to CDC (Dr. Bernard Beall) for verification and subsequent designation of *emm* subtype.

Ethical considerations. The study was approved by the Faculty of Medicine, Addis Ababa University, and the Armauer Hansen Research Institute/All African Leprosy Rehabilitation and Training Centre Ethical Review Committees and National Ethical Review Committee of Ethiopia. Furthermore, written informed consent and verbal assent were obtained from each child's parent or guardian and from all study participants respectively.

RESULTS

Table 1 presents the *emm*/st types and geographic origins of the isolates. The 82 isolates examined represented 47 different sequence types. Among them, 20 GAS isolates belonged to 13 new *emm* subtypes, never previously reported in the world.

Six different types—*emm* 3.19, *emm* 5.48, st62.0, *emm* 29.2, st463.0, and st3757.0, listed in descending order of frequency—made up 35.4% of the isolates (table 1). The most frequent type, *emm* 3.19, constituted 14.6% of the total isolates. Previously undocumented *emm* 5.48, one of the most common types in our collection, was isolated only from Gondar.

There was no significant overlap in distribution of GAS types in the 3 study sites (table 1). Only 9 *emm* types were isolated from 2 study sites. Addis Ababa and Gondar shared many of the overlapping GAS types, specifically *emm* 3.19, *emm* 28.0, *emm* 38.1, *emm* 106.2, and st463.0. In Addis Ababa and Dire-Dawa, *emm* 74.0 and st1731.1 were overlapping, whereas Gondar and Dire-Dawa had st3757.0 and st430 in common. However, no isolate was detected in all 3 study sites.

It is noteworthy that among the 82 isolates in our collection, only 37 isolates, representing 14 sequence types, are included in the 26-valent GAS vaccine, and the other 45 isolates, representing 29 sequence types, are not included (table 2).

DISCUSSION

Developing countries have the highest documented rates of rheumatic fever worldwide. To design effective preventive strategies, it is vital that we understand the molecular epidemiology of GAS in these countries. We undertook an *emm*-based analysis of 82 GAS isolates from 3 different geographic locales in Ethiopia: Addis Ababa (central), Gondar (northern), and Dire-Dawa (southeastern). This study possibly represents the first large-scale genotypic survey of GAS isolates among schoolchildren in Africa.

The 82 isolates examined represent 43 different sequence types, a diversity that is higher than found in several *emm* typing reports from other countries [28–30]. Among our collection, 18 isolates belong to 13 new, previously undocumented subtypes. In this study, 6 types (*emm* 3.19, *emm* 5.48, st62.0, *emm*29.2, st463.0, and st3757.0) accounted for 35.4% of isolates. Similarly, an earlier study assessing *emm* diversity of isolates collected in 1990 detected 49 distinct *emm*/st types among 90 isolates from throat cultures of healthy carriers [25]. A predominance of particular GAS types in both studies is therefore less than that seen in other studies. In Japan, only 29 *emm*/st types were detected among 906 clinical isolates [31]. In Mexico, only 31 *emm* types were detected among 423 isolates [29]. Six serotypes accounted for 60% of all isolates recovered from US patients with uncomplicated pharyngitis [32].

Few reports examining *emm* type diversity outside of Western nations are available; however, most studies support the presence of dominant types. A community study in Korea examining community-wide GAS strain diversity found that *emm* 78 and *emm* 23 accounted for 69% of GAS isolates in one region, whereas in another region, 4 types accounted for 52% of isolates [33]. By contrast, studies of isolates collected in Australia and India revealed much greater diversity of GAS strains [20, 34]. The factors that determine the prevalence of

Table 2. *emm* Types included in the multivalent vaccine composed of 26 *emm* types.

emm Types in vaccine	Represented among Ethiopian isolates	Addis Ababa	Gondar	Dire-Dawa	Total no. of isolates
emm 1	Yes	2	0	0	2
emm 1.2	No	…	…	…	…
emm 2	Yes	2	0	0	2
emm 3	Yes	11	1	0	12
emm 5	Yes	2	4	0	6
emm 6	Yes	1	0	0	1
emm 11	No	…	…	…	…
emm 12	Yes	0	…	1	1
emm 13	No	…	…	…	…
emm 14	Yes	0	1	0	1
emm 18	Yes	1	0	0	1
emm 19	No	…	…	…	…
emm 22	No	…	…	…	…
emm 24	No	…	…	…	…
emm 28	Yes	2	1	0	3
emm 29	Yes	4	0	0	4
emm 33	No	…	…	…	…
emm 43	Yes	0	1	0	1
emm 59	No	…	…	…	…
emm 75	Yes	1	0	…	1
emm 76	No	…	…	…	…
emm 77	No	…	…	…	…
emm 89	Yes	0	0	1	1
emm 92	Yes	1	0	0	1
emm 101	No	…	…	…	…
emm 114	No	…	…	…	…
Total no. of *emm* types represented/no. of isolates	…	10/27	5/8	2/2	14/37

diversified GAS types in such areas and the predominance of certain *emm* types in others deserve further study.

The epidemiological patterns show differences among the current study sites, regardless of the small number of sampled isolates from each city. For example, our data showed that in Addis Ababa, *emm* 3.19 is a major type among the isolates. However, only one such strain was identified in Gondar and none in Dire-Dawa. In Gondar, *emm* 5.48 and st62.0 were the most predominant, accounting for 14.7% each (4/23). There was no overlap in the distribution of GAS types in the 3 study sites, except for 7 isolates. Five of the 7 overlaps *(emm* 3.19, *emm* 28.0, *emm*38.1, *emm* 106.2, and st463.0) occurred in Addis Ababa and Gondar. This may show that the 2 sites may have a sort of resemblance in GAS epidemiology, more than each of them does with Dire-Dawa. Although a clear reason for this observation is not apparent, it may partly be explained by the high influx of population from the northern part (including Gondar) of Ethiopia to Addis Ababa in previous years due to civil war and recurrent famine. The authors suggest that methods such as PFGE be applied to determine the relatedness of isolates from the cities.

According to the present study, type *emm* 3 was the most frequent in pharyngeal isolates of healthy schoolchildren in Addis Ababa, a type that was disproportionately represented in invasive diseases elsewhere [32, 35–38]. Although, carriage of *emm* 3–type GAS is quite high in Addis Ababa, severe invasive disease due to GAS, such as streptococcal toxic shock–like syndrome and necrotizing fasciitis, are not reported to the best of our knowledge. This may be owing to poor diagnostic facilities available. The implications of a high rate of *emm* 3 GAS carriage in Addis Ababa on severe invasive diseases are worth exploring.

The concept of distinct throat and skin *emm* types has been widely accepted. Two *emm* types with 3 isolates each, *emm* 80 and st212, were associated with skin origin only in the previous study carried out in Addis Ababa [25]. Conversely, both of these types (1 each) were isolated from throats of healthy schoolchildren in this study.

The current study has limited ability to represent the larger

population, particularly rural communities; nevertheless, it is reasonable to suggest that the highly diversified nature of the GAS population demonstrated in this and the previous study may challenge the application of a multivalent vaccine composed of the N-terminal region of the M-protein. A multivalent vaccine composed of 26 M-protein N-terminal regions was anticipated to prevent ~90% of invasive GAS infections in the United States [39]. Of the 26 *emm* types included in this multivalent vaccine, only 57.4% of isolates in Addis Ababa, 34.8% in Gondar, and 16.7% in Dire-Dawa were of these types. In addition, 10 (59%) of the 17 most frequently isolated *emm* types in the present study are not included in the vaccine. Although the sample isolates may not represent the larger population and were collected only from healthy children, it is reasonable to hypothesize that the vaccine would protect only 45% from infection if applied in the study area.

A similar *emm* type survey conducted on isolates collected in 1990 from healthy schoolchildren (Addis Ababa) showed that *emm* 12 was the predominant sequence type, accounting for 23% (9 of 39) of all isolates [25], whereas in this study, *emm* 3.19 was the most common type, with 23% (11 of 47) prevalence in Addis Ababa. In addition, of 20 *emm* types demonstrated in 1990, only 3 sequence types (15%)—*emm* 1, *emm* 28.0, and *emm* 106.2—were represented in this study. Examples of changes in the prevalent strains have been reported elsewhere. A 2-year intensive study with weekly or biweekly cultures from children revealed the gradual introduction and increase in prevalence and retention of specific serotypes. In that report, the sudden appearance and rapid disappearance of a single serotype was noted [40]. Likewise, observation in this study demonstrates possible replacement of *emm* types in Addis Ababa; however, the rate of replacement with new *emm* types is not known and warrants further study.

These findings emphasize that even if all the currently circulating *emm* types are covered, it is still possible that new strains not covered by a vaccine could be introduced into the vaccinated population and that naive hosts could become colonized [40]. Thus, the 26-*emm* type multivalent vaccine may prove to be ineffective in preventing GAS infection in the Ethiopian population. We therefore suggest that other prime vaccine candidates, such as C5a peptidase, fibronectin-binding proteins, and M-protein conserved region vaccines should be studied to address the vaccine requirements in this region [41–43].

In conclusion, we have shown that among Ethiopian schoolchildren, there are a large number of circulating GAS *emm* types. The 3 sites studied demonstrated significant difference in the distribution of GAS types. The circulating types of GAS in a community may be replaced with others through time. Fifty-five percent of the isolates circulating in Ethiopian schoolchildren were not included in the 26-valent M-protein vaccine.

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Potential conflicts of interest. All authors: no conflicts.

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