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Longitudinal Analysis of Group A *Streptococcus emm* Types and *emm* Clusters in a High-Prevalence Setting: Relationship between Past and Future Infections

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(See the Editorial Commentary by Beall and Van Beneden, on pages 1394–7.)

Group A *Streptococcus* is a pathogen of global importance, but despite the ubiquity of group A *Streptococcus* infections, the relationship between infection, colonization, and immunity is still not completely understood. The M protein, encoded by the *emm* gene, is a major virulence factor and vaccine candidate and forms the basis of a number of classification systems. Longitudinal patterns of *emm* types collected from 457 Fijian schoolchildren over a 10-month period were analyzed. No evidence of tissue tropism was observed, and there was no apparent selective pressure or constraint of *emm* types. Patterns of *emm* type acquisition suggest limited, if any, modification of future infection based on infection history. Where impetigo is the dominant mode of transmission, circulating *emm* types either may not be constrained by ecological niches or population immunity to the M protein, or they may require several infections over a longer period of time to induce such immunity.

Keywords. *Streptococcus pyogenes*; *emm* cluster; immunity; skin infection.

Group A *Streptococcus* (GAS) is a pathogen of global importance, responsible for >700 million superficial infections and ≥500 000 deaths per year [1, 2]. Almost all diseases caused by GAS are most common in developing regions, from superficial conditions, such as pyoderma (including impetigo) and pharyngitis, to severe sequelae, including invasive disease, rheumatic heart disease, and poststreptococcal glomerulonephritis [1]. Despite the ubiquity of GAS, our understanding of its immunobiology and the relationship between infection, colonization, and immunity remain incomplete, hindering efforts at sustainable control, including the development of safe and effective vaccines.

The GAS M protein is a major virulence factor that elicits antibody production and enables the bacteria to inhibit phagocytosis in the absence of antibodies, making it a prime vaccine candidate [3]. This protein forms the basis of a number of classification systems for GAS: *emm* typing, *emm* patterns, and *emm* clusters, where *emm* refers to the *emm* gene encoding this surface M protein [4]. More than 240 *emm* types have been identified based on the variable N-terminus part of the protein, contributing to the complexity of epidemiologic studies [5]. Based on the structure of *emm* and *emm*-like genes in the GAS genome, *emm* types may be further grouped into *emm* patterns, referred to as A–C, D, and E [6].

Recently, *emm* types have been grouped into 48 *emm* clusters based on closely related sequences, shared structural characteristics, and similar binding capacities [7, 8]. It has been hypothesized that cross-protective immunity may occur between *emm* types that exist within the same *emm* cluster. Preliminary and limited laboratory studies have shown that in vitro cross-protection does occur within certain *emm* clusters in Fijian children [8]. The existence of cross-protective immunity within *emm* clusters could substantially aid vaccine development against this multistrain pathogen. However, it remains to be seen whether these findings translate to population-level protection.

The relationship between *emm* types and disease burden is important when selecting priority strains for prevention. It

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has long been believed that different GAS strains preferentially cause either impetigo or pharyngitis [9]. Based on a number of population-based surveys, GAS strains with the *emm* pattern A–C display a tropism for the throat, whereas D have a tropism for the skin and E are found in both tissue sites [6, 10]. However, the mechanism responsible for different disease manifestations remains to be identified [11]. The distributions of *emm* types and clinical manifestations differ between settings, with fewer *emm* types circulating in low-prevalence settings (typically dominated by pharyngitis) than in high-prevalence settings (typically dominated by impetigo), and with some *emm* types common in developed countries rarely found in developing countries [12–14].

In the current study, we investigated longitudinal data on *emm* types, patterns, and clusters at individual, school, and regional levels to evaluate *emm* immunobiology within a streptococcal disease endemic setting. We examined *emm* types isolated from children over time for evidence of immune protection at either the *emm* type or *emm* cluster level. First, we hypothesized that if immunity to prevalent *emm* types did develop, we would observe the subsequent disappearance of these *emm* types at individual and population levels as opportunities for further transmission diminished, followed by the appearance of new *emm* types to which the population had no prior immunity. Second, we also hypothesized that if immunity to *emm* types did develop, we would observe different *emm* types circulating in children infected at a single time point than in children infected at multiple time points, because immunity would limit the acquisition of *emm* types that could cause a subsequent infection. Third, we framed similar hypotheses in terms of *emm* clusters.

METHODS

Ethical Approval

This study was approved by the Fiji National Research Ethics Review Committee, the Fiji National Health Research Committee, and the University of Melbourne Human Research Ethics Committee. Written informed consent was required from participants or from a parent or guardian before collection of information.

Setting and Participants

This was a prospective longitudinal cohort study conducted in 3 schools in the Central Division of Fiji from February 2006 to November 2006. Two of the schools were rural, with all children of iTaukei (Indigenous Fijian) ethnicity, and the third school was located in Suva, with most children of Indo-Fijian ethnicity. Enrollment rates for the 3 schools were 96.4% (rural school 1), 80.6% (rural school 2), and 53.2% (urban school) [15].

For skin screening, each school was visited every 2 months over a 10-month period, with a total of 6 visits per school. Children aged 5–15 years were screened for skin sores, and swab samples were obtained from crusted or purulent sores [15]. At the first skin screening visit, children without sore throat symptoms had their throats swabbed for evidence of asymptomatic colonization [16]. Over the 10-month period, each school was visited twice per week, and throat swab samples were collected from children reporting sore throat symptoms within the preceding 7 days [16].

Laboratory Methods

Skin and throat swab samples were collected, transported, and tested for the presence of β-hemolytic colonies using standard methods [15, 16]; *emm* typing of the dominant β-hemolytic colony was undertaken according to US Centers for Disease Control and Prevention standard methods [15–17].

Data Preparation and Analysis

Characterization and Analysis of emm Types

Data were restricted to Lancefield group A isolates, and *emm* types were assigned to *emm* clusters per Sanderson-Smith et al [7] and analyzed by time period, school, disease type, and participant. Each skin screening visit took place during a period of approximately 2 weeks, followed by a window of about 6 weeks before the next round of skin screening visits began. The study period was therefore divided into 6 screening time periods, including 5 of approximately 2 months' duration, each running from the date of the first skin screening associated with that visit to the day before the first skin screening associated with the next visit, and a sixth visit of approximately 2 weeks' duration, covering the final skin screening visit. Throat isolates were assigned to the screening period within which they were collected.

Diversity and Prevalence

At the child level, the number of distinct *emm* types and *emm* clusters was tabulated against the number of positive swab samples. The Simpson index of diversity, the probability that 2 randomly selected *emm* types are different, was calculated for each screening period by school [18].

The total number of isolates of each *emm* cluster and each *emm* type were calculated. The prevalence of each *emm* type per 1000 children was calculated at each time point by dividing the number of isolates by the number of children participating in that visit, stratified to the same level. The *emm* clusters were ordered by the highest number of samples overall, and within each cluster, *emm* types were similarly ordered.

Individual-Level Exposure Responses

Children with >1 isolate of the same *emm* type during the study were identified and their screening, pharyngitis and *emm* type history reported, noting that the absence of an isolate for a screening visit may indicate that no sores were present, sores were present but no swab sample was obtained (because sores were not crusted or purulent, as specified in the study protocol), or a swab sample was obtained but GAS did not grow. For these

children, we also report the *emm* clusters corresponding to their *emm* types, in the Supplementary Material.

The ordering of *emm* types and *emm* clusters from highest to lowest number of isolates was compared between 2 groups of children: those with a single positive isolate and those with multiple positive isolates. Data were prepared using Stata (release 14; StataCorp) and MATLAB 2017b (The Mathworks) software, and analyses were conducted using R software (version 3.4.4).

RESULTS

Demographics

A total of 457 children were enrolled in the study, with a minimum of 400 seen at any of the 6 skin screening visits and 80% of children seen at all of them. The number of children screened per visit was 73–80 and 160–175 for rural schools 1 and 2, and 161–202 for the urban school. All 255 children enrolled from rural schools 1 and 2 identified as iTaukei (Indigenous Fijian); the population at the urban school included of 99 Indo-Fijian children (49%), 67 (33%) iTaukei, and 36 (18%) of other ethnicities. The sexes were evenly distributed, with 229 of the 457 children female. The children's median age was 9.9 years (interquartile range, 7.9–12.0 years).

Clinical Data

There were 451 GAS-positive isolates collected from 245 children during the study. Of these, 379 (84%) were from impetigo samples, 45 (10%) from pharyngitis samples, and 27 (6%) from asymptomatic throat colonization samples, which were collected only at the first visit. The median prevalence of GAS infection per 1000 children per screening period (excluding colonization) was highest in rural school 2 (227.5; interquartile range, 199.2–247.4), followed by rural school 1 (167.6, 135.1–237.5) and the urban school, with the lowest observed prevalence (97.0, 78.4–135.5).

Of the 245 children with GAS-positive isolates, most (195 [80%]) had either 1 or 2 GAS-positive swab samples (Figure 1A). The highest number of GAS-positive swab samples (impetigo, pharyngitis, and asymptomatic throat colonization combined) for any child was 6, in 2 children (<1%).

Diversity and Prevalence

The 5 most frequently observed *emm* types were *emm*70, *emm*33, *emm*25, *emm*93.3, and *emm*11, accounting for about 30% of positive isolates. This ranking held both for children infected only once and for children infected more than once. The 5 most frequently observed clusters were D4, E3, E6, E4, and E2, for children infected only once and for children infected more than once, accounting for 77% of positive isolates in both groups.

Of the 128 children with >1 GAS-positive swab sample, 100 had a different *emm* type and 83 had a different *emm* cluster isolated from each of their swab samples. Most children with 2 positive swab samples had 2 different *emm* clusters (65 of 78 children [83%]) rather than a single cluster. Most children with ≥3 positive swab samples had ≥1 repeated *emm* cluster (36 of 50 children [72%]) (Figure 1B).

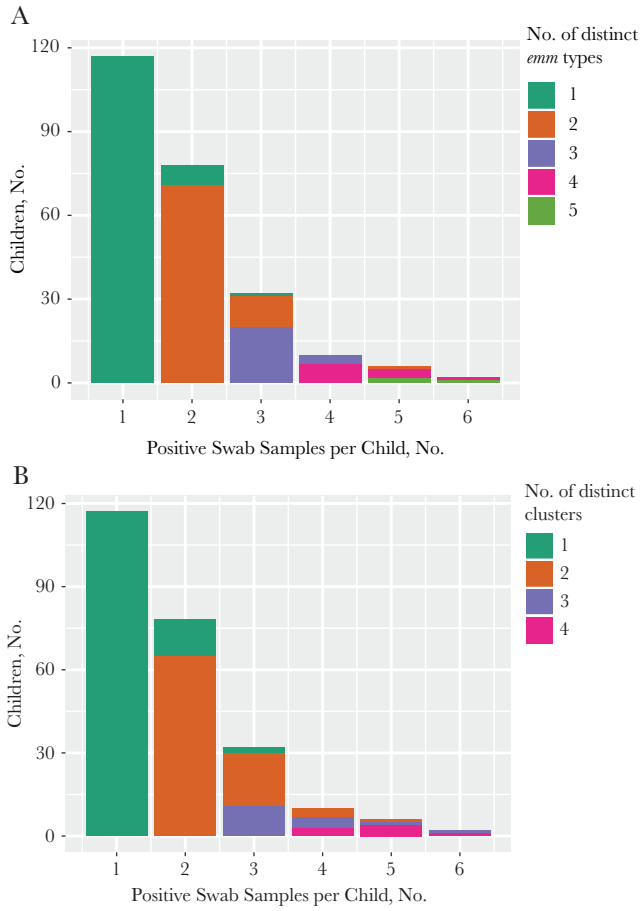

Figure 1. Numbers of group A *Streptococcus*–positive swab samples per child, colored according to the number of distinct *emm* types (*A*) or distinct *emm* clusters (*B*) present in these samples.

A wide variety of *emm* types circulated during all 6 screening periods (range, 19–37 *emm* types per screening period). There was no evidence of prevalent *emm* types disappearing, followed by the appearance of new dominant *emm* types (Figure 2). Rather, the most prevalent *emm* types were consistently present in a given setting (eg, *emm*70 and *emm*33), and less prevalent *emm* types were detected during only 1 or 2 screening periods. There was no evidence of competitive exclusion of *emm* types at the population level, with each *emm* cluster having several isolates circulating concurrently in the same setting. Urban and rural schools seemed to have different patterns of circulation, with *emm*70 highly prevalent in both rural schools throughout the entire study but completely absent from the urban school. In contrast to the rural schools, there were no dominant *emm* types at the urban school, and no *emm* types consistently isolated across all screening periods.

Despite differences in the overall prevalence of GAS-positive swab samples for each screening period, high diversity was observed

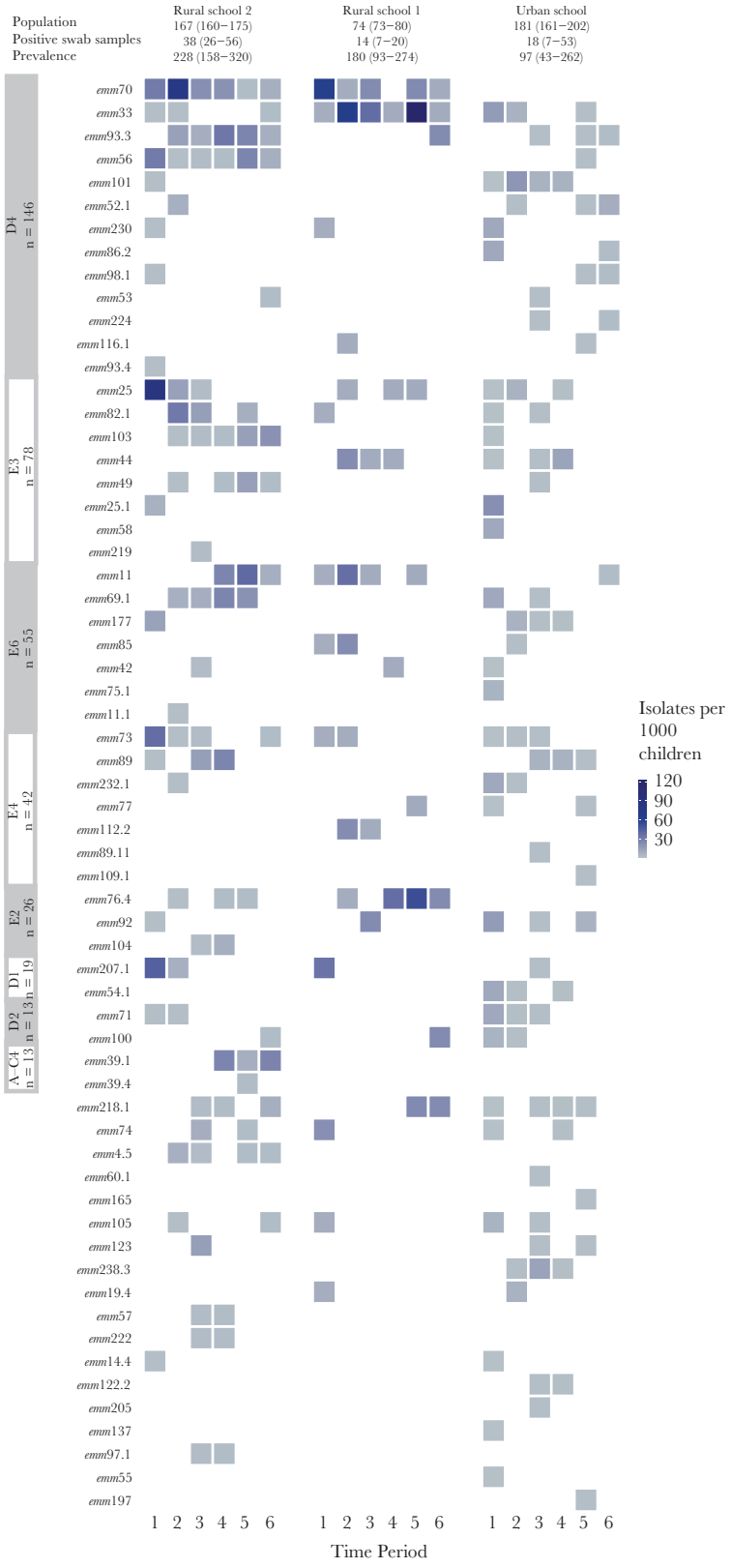

Figure 2. Prevalence of *emm* types per 1000 children for each screening time period, grouped by cluster and stratified by school. The *emm* clusters (labeled on y-axis) are ordered by the overall number of isolates, and within each *emm* cluster, *emm* types are also ordered by overall number of isolates. Data for each school include the median population, number of positive swab samples, and overall prevalence, together with their ranges.

across both rural and urban settings (Figure 2), with the Simpson index of diversity ranging from 0.77 to 0.99 (Supplementary Figure 1). No clear association was observed between prevalence and diversity, and we did not perform a formal test of association, given the small number of data points and observed differences per setting. The prevalence of infection by cluster related directly to the number of *emm* types categorized within each cluster. For example, the most prevalent cluster in our study was D4, which is the largest cluster with 32 *emm* types. The next most prevalent cluster was E3, which is the second largest cluster with 19 *emm* types (Supplementary Figure 2).

The vast majority of *emm* types (56 of 62 detected) were recovered from impetigo samples (Figure 3). Even for those *emm* types consistently present throughout the study period, isolation from pharyngitis was rare and generally not repeated over >2 consecutive screening periods (with the exceptions of *emm*44, *emm*92 *emm*101, and *emm*238.3). Throat colonization isolates collected at the first skin screening visit did not greatly increase the *emm* type diversity, adding only 2 *emm* types (*emm*137 and *emm*14.4). Whereas cluster D4, typically associated with skin infection, dominated the impetigo isolates, it was also present in pharyngitis isolates during each screening period, along with cluster E3 (Supplementary Figure 3). Throat colonization isolates collected at the first skin screening visit were mostly from the clusters with the highest prevalence of skin isolates at that time, with *emm* pattern D comprising 63% of the colonization isolates (Supplementary Table 1). Conversely, *emm* pattern A–C, typically associated with throat infection, was isolated from about 16% of pharyngitis isolates.

Individual-Level Exposure Responses

For the 28 children with repeated isolation of the same *emm* type, a variety of longitudinal patterns was observed (Figure 4). Four children (children 9, 11, 13, and 16) had the same *emm* type isolated from pharyngitis and impetigo samples at different times, and 3 (children 22, 24, and 27) had the same *emm* type isolated from throat colonization and impetigo samples on the same day. For skin infections, reacquisition of the same *emm* type after a documented skin screening without GAS infection was observed in 6 children (children 1, 10, 17, 18, 19, and 28). Two different *emm* types were isolated from impetigo samples obtained on the same day in 4 children (children 2, 4, 7, and 19). For these same 28 children (Supplementary Figures 4 and 5), we observed different *emm* types from the same cluster isolated at the same time (child 4), and at different times (children 1, 2, 4, 7, 17, and 26). In addition, 3 of the 28 children had *emm* types from different clusters isolated concurrently (children 2, 7, and 19).

DISCUSSION

Our analysis of longitudinal GAS *emm* types, patterns, and clusters in Fijian schoolchildren provides insights into the links between prevalence, diversity, and tissue tropism. We found no evidence of tissue tropism in this setting, with the *emm* types isolated from pharyngitis and throat colonization samples reflecting those isolated from impetigo samples, the dominant mode of infection in this cohort. There was no evidence of displacement of one *emm* type by another over time, with common *emm* types generally present throughout the study. There was no apparent selective pressure or constraint of *emm* types within clusters, with multiple *emm* types from each cluster circulating concurrently. Despite differences in prevalence by site and by time, high levels of diversity were observed in both urban and rural settings.

The patterns of acquisition in the small number of children with the same *emm* type isolated more than once suggest limited, if any, modification of future infection based on infection history in these children. It may be that the small number of children experiencing repeated infection with the same *emm* type indicates that most children developed immunity to the *emm* type that caused their infection. We cannot be certain whether the limited number of repeated infections we observed is due to immune protection or a lack of reexposure to the same *emm* type. Our observation that the same *emm* types and clusters circulated in children infected at single and multiple time points raises questions about the immunity induced by the M protein in this tropical setting and its capacity to limit the collection of *emm* types or clusters that could cause subsequent infections. It is important to note that our conclusions regarding individual and population immunity to GAS are based on infection patterns rather than measurement of M type–specific or other antibodies to GAS. However, measurement and understanding of the GAS immune response is not straightforward, with inconsistent development of antibodies after acquisition and a lack of evidence for a protective effect [19–21].

A particular strength of our study is the novel longitudinal analysis of *emm* types and clusters, with limited loss to follow-up. More than 80% of children in our cohort were seen at all 6 skin screening visits, with swab samples obtained from all crusted and purulent skin sores, providing rich data on the bacterial population. As with all studies, there are limitations. With a likely resolution time of <1 month (Adrian Marcato, The Peter Doherty Institute for Infection and Immunity, personal communication), we may have missed detecting some impetigo infections that arose and resolved within our 2-month screening period. Additional infections could add to the level of observed strain diversity, making our estimates a lower bound of diversity.

Furthermore, although children may have had as many as 2 swab samples collected from different impetigo lesions, only the dominant colony from each sample was *emm* typed. We may have missed detecting multiple *emm* types within a single lesion, again meaning that our results may underestimate overall diversity. The sporadic detection of many *emm* types in our age-limited population sample suggests that the relevant mixing

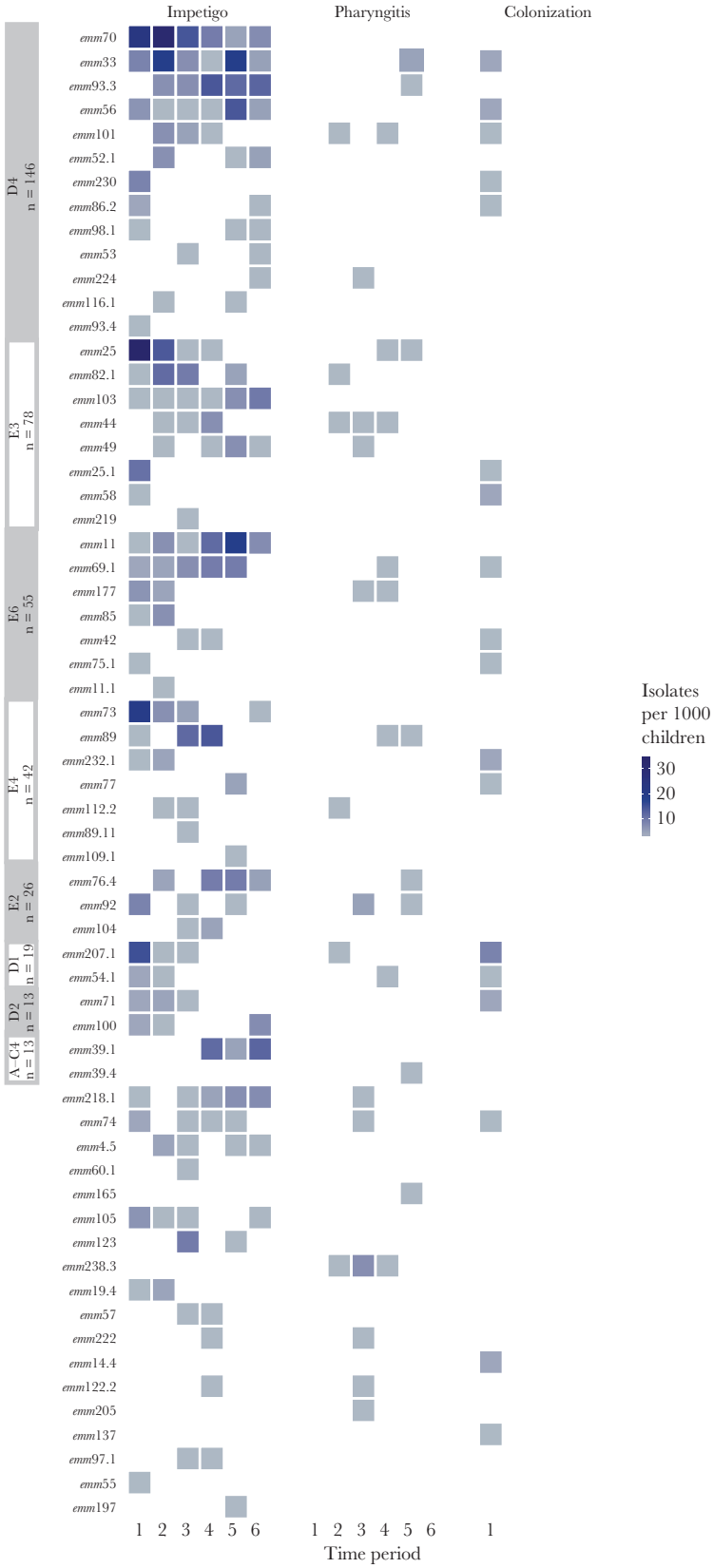

Figure 3. Prevalence of *emm* types per 1000 children for each screening time period, grouped by cluster and stratified by specimen type (impetigo, pharyngitis, or colonization). The *emm* clusters (labeled on y-axis) are ordered by the overall number of isolates, and within each *emm* cluster, *emm* types are also ordered by the overall number of isolates.

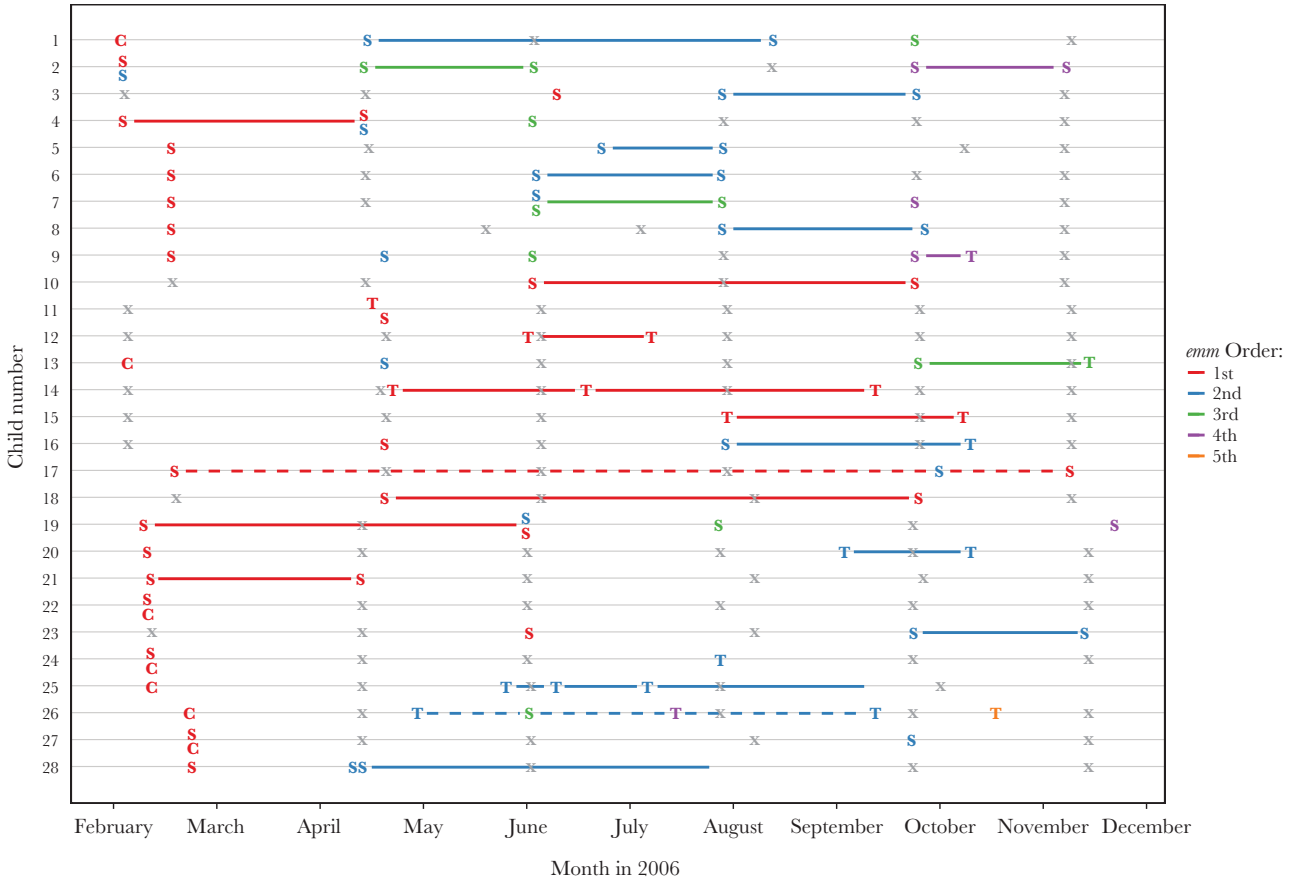

Figure 4. Longitudinal information for the subset of 28 children with repeated isolation of the same *emm* type. Solid lines connect the same *emm* type isolated more than once; dotted lines, different *emm* types isolated in the 2 samples. Colors represent unique *emm* types, with symbols representing the type of sample (S, skin; T, throat [with symptoms]; and C, throat [without symptoms]). Each *x* represents a skin screening visit attended by the child during which no group A *Streptococcus* (GAS) was isolated, either because no sores were present, sores were present but no swab sample was obtained, or a sample was obtained but GAS did not grow.

pool is wider than schools, and infection may be readily acquired elsewhere in the community, including households. However, because only symptomatic infections were swabbed after the first skin screening visit, we were unable to investigate any relationship between throat colonization and skin infection, and these *emm* types may have been present asymptomatically in our cohort throughout the study. It is possible that GAS recovered from throat swab samples during pharyngitis episodes was also asymptomatic colonization. For the single time point with throat colonization data, all but 2 of the *emm* types found in asymptomatic children were also present in impetigo and/or pharyngitis isolates. However, without longitudinal data on asymptomatic colonization in this cohort, we cannot estimate the overall contribution of colonization to strain diversity.

The large number of concurrently circulating *emm* types (between 19 and 37 types per screening period) is consistent with observations from other settings with a high prevalence of impetigo [13, 22–24]. Studies in a mouse model of skin infection suggest that reinfection with the same *emm* type within a short period is required to stimulate type-specific immunity after skin infection [25]. If this requirement holds true for human infections, this phenomenon would be anticipated to positively select for diversity in high-prevalence settings, because a large number of circulating *emm* types makes reexposure to the same *emm* type less likely. The observation that very few children (28 of 457 [6.1%]) had repeated isolation of the same *emm* type within the study period suggests that there would be limited opportunity for the development of type-specific immunity, should reexposure be necessary. In an earlier study in a low-prevalence population, where colonization was the dominant source of GAS isolates and pharyngitis the dominant disease manifestation, children were equally likely to acquire the same *emm* type as to acquire a different *emm* type in a single year [26]. This observation further supports the notion that low-prevalence/low-diversity settings afford greater opportunity for the development of natural immunity, given the greater likelihood of reexposure to the same strain.

We did not observe the classic epidemiologic picture of A–C pattern strains dominating pharyngitis isolates and pattern D representing a very small percentage of pharyngitis isolates [6].

Rather, we observed patterns D and E dominant for both impetigo and pharyngitis (and pattern D dominant for colonization), consistent with studies in other high-prevalence settings [12–14, 27, 28]. Causal mechanisms for this tropism remain uncertain [6, 29], but they seem to be overwhelmed by transmission pressure in high-prevalence settings.

An in vitro study of a subset of the children in our analysis found that skin infection may elicit a functional immune response in some children with M type–specific and cross-reactive immune responses after skin infection [8]. However, results varied depending on the *emm* cluster analyzed, and the evidence was not strong for the D4 cluster, the most prevalent in our population [8]. Our observations of reacquisition of the same *emm* type or cluster during the period of our study suggest that the duration of protective immunity after infection may be short or even absent in some children. One could hypothesize that other, non–M protein, GAS antigens may be needed to protect against GAS infection owing to D4 cluster in particular, and owing to skin-associated *emm* types in a tropical setting in general. A GAS vaccine would definitively need to provide a substantially longer duration of protection than that we observed for some children to have an impact on prevalence.

This longitudinal study suggests that in settings where impetigo is the dominant mode of transmission, circulating *emm* types either may not be constrained by ecological niches or population immunity to the M protein or may require several infections over a longer period of time to induce such immunity. With limited evidence of M immunity to GAS apparent in our data, further work is needed to understand how settings have transitioned from high to low prevalence (as has happened in many developed countries), because this may provide clues for future control.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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