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ORIGINAL ARTICLE

Long-term, single-center surveillance of non-invasive group A streptococcal (GAS) infections, *emm* types and *emm* clusters

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Abstract

Group A streptococci (GAS) are among the most frequent pathogens in children. Many epidemiological studies focus on specific GAS infections (such as tonsillopharyngitis or invasive disease), on GAS carriers or on post-streptococcal sequelae. By comparison, reports on regional GAS characteristics, particularly circulating non-invasive GAS in Europe, are rare. In a monocentric study, all GAS isolated from pediatric patients at a tertiary care hospital over a 6-year period (2006–2012) were characterized. GAS e*mm* types and clusters were determined. Associated patient data were analyzed. Five hundred sixty-six GAS strains were collected. GAS tonsillopharyngitis was most common (71.6%), followed by pyoderma (6.0%), otitis media (3.7%), perineal dermatitis (3.4%), and invasive infections (1.4%). Colonizing strains represented 13.6% of GAS. GAS e*mm*12 was most prevalent among invasive and non-invasive isolates. *Emm*1, *emm*4, *emm*28, and *emm*89 were the most frequent non-invasive GAS strains. The *emm* E4 cluster was most common, followed by the A-C4, A-C3, and E1. Among the GAS infections, different *emm* types and clusters were identified, e.g., *emm4* was more common among patients with scarlet fever. Three new *emm* subtypes were characterized: *emm*29.13, *emm*36.7, and *emm*75.5. This comprehensive review of a large, local GAS cohort points to the differences between and similarities among GAS genotypes and disease manifestations, while minimizing regional variations. Considerable deviation from previous epidemiological findings is described, especially regarding the frequent detection of *emm1* and *emm89* in non-invasive GAS infections. Periodic updates on molecular and epidemiological GAS characteristics are needed to track the multifaceted pathogenic potential of GAS.

Keywords Group A streptococci (GAS) · *Streptococcus pyogenes* · Epidemiology · Pediatric infections · Circulating non-invasive isolates

Introduction

Invasive GAS infections and post-streptococcal sequelae significantly contribute to human morbidity and mortality worldwide [1–3]. National surveillance reports [4–10] and multinational studies [11–14] periodically have characterized the molecular epidemiology of invasive GAS infections, but fewer studies have focused on the long-term evaluation of non-invasive GAS infections in Europe [15–18]. Non-invasive GAS infections range in frequency and can include acute tonsillopharyngitis (with and without scarlet fever), otitis media, pyogenic skin infections (such as impetigo contagiosa), vulvovaginitis, balanoposthitis, and recurrent perineal dermatitis [1, 9]. The global burden of localized GAS skin and throat infections was estimated to exceed 111 million cases per year, resulting in considerable economic, social, and medical care challenges [1].

One of the major GAS defense mechanisms countering the human immune system is the M protein [19–21]. Anchored to the surface of GAS, it facilitates the evasion of phagocytosis by polymorphonuclear leukocytes [22] and induces a type-specific antibody response [19]. An increase in M-based immunity and cross-reactive immune response against GAS has

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been shown in both invasive and non-invasive GAS infections [19, 21, 23].

Since the M protein was first described in 1928, typing of its gene sequence *emm* and subsequent grouping into *emm* clusters often has been used to characterize the epidemiology of GAS [24–27]. The ten most common *emm* types collected through the Strep-EURO program were, in decreasing prevalence order, *emm1*, *emm28*, *emm3*, *emm89*, *emm87*, *emm12*, *emm4*, *emm83*, *emm81*, and *emm5* [14]. Whereas *emm1* and *emm3 most commonly* were found in streptococcal toxic shock syndrome and necrotizing fasciitis, *emm81* and *emm77* were more prevalent in patients with local GAS infections. However, other global epidemiological studies [12, 21, 28] have shown that the distribution of *emm* types can vary widely among countries, as well as among specific clinical manifestations. In addition to natural variation among predominant *emm* types, variation among countries also has been assumed to reflect a population's susceptibilities to particular GAS strains [18]. Additionally, outbreak reports of invasive GAS infections often have been associated with specific GAS clones, with *emm1* [14, 29] and *emm89* [16, 30, 31] raising concern about particularly virulent GAS strains. Currently, it is unclear whether the emergence of certain GAS strains should be attributed to their virulent potential or whether it simply may reflect their widespread distribution within a population [32].

Therefore, the current study focused on the regional prevalence of GAS infections (invasive and non-invasive), as well as on GAS colonization detected during routine clinical care of patients who sought treatment at a tertiary care pediatric hospital in Freiburg, Germany. Using a long-term, monocentric study design, the frequency of GAS colonization, as well as of invasive and non-invasive GAS infections, was evaluated. In a second step, GAS *emm* types and *emm* clusters were determined. Their longitudinal developments were then analyzed with regard to annual or seasonal changes and in relation to potential outbreak situations during the study period. In a last step, the associations of *emm* types and *emm* clusters with GAS disease manifestations were examined.

Methods

Study cohort

During the study period (March 11, 2006, to May 19, 2012), all patients with GAS-positive microbiological cultures were included in the study. As part of routine clinical diagnostics, microbiological cultures were taken from blood, urine, cerebrospinal fluid (CSF), and joint and pleural fluid aspirates, as well as from throat, skin/wounds, meatus acusticus externus, and from the genital/perineal region. Microbiological diagnostics were conducted at the bacteriological laboratory of the Center for Pediatrics and Adolescence Medicine at the University Medical Center Freiburg, Germany. In the event of GAS positivity, GAS were isolated and aliquots stored at −20 °C, before being shipped (with dry ice and stored at −80 °C) to the laboratory of the infectious disease group at the Clinic and Policlinic for Pediatric and Adolescence Medicine, Carl Gustav Carus University Medical Center Dresden, Germany. Clinical data retrospectively were obtained from patient charts. A subgroup was defined as presumable GAS carriers by the combination of (a) having a GAS-positive throat swab without also having a clinical diagnosis of tonsillopharyngitis, or (b) presenting with signs of respiratory tract infection (including tonsillopharyngitis but predominantly cough, rhinitis, croup, and bronchitis), obtaining a McIsaac score of ≤ 2 and achieving clinical restitution without antibiotic treatment. The presumption of a rather viral infection was made by the clinician in charge, sometimes also based upon a positive multiple PCR result for respiratory syncytial virus, adenovirus, influenza virus A/B or parainfluenza virus 1–4, and/or a differential blood count or blood biochemistry (e.g., C-reactive protein). Since none of these criteria provide proof for the diagnosis of a viral infection with GAS carrier state versus the diagnosis of GAS tonsillopharyngitis, we named this subgroup "presumable GAS carriers." This was in accordance with the final diagnosis of the attending physician.

GAS cultures

Isolates identified as GAS according to standard microbiological procedures were streaked on Columbia agar with 5% sheep blood (bioMerieux) and incubated overnight at 37 °C in the presence of 5% CO_2. GAS positivity of these ß-haemolytic monocultures was confirmed by latex agglutination of the streptococcal Lancefield antigen A (Slidex Strepto Plus, bioMerieux). One GAS colony was additionally incubated overnight in liquid Todd-Hewitt-Broth-Medium (THB) for enrichment before DNA isolation. If the initial overnight culture did not show GAS growth, re-culturing of the shipped GAS aliquots was repeated three times using an extended culture time. Study cases with negative GAS cultures, as defined at day 7 of incubation, were excluded from further analysis. For details regarding the study cohort, please refer to supplementary Figure 1.

Molecular GAS typing

DNA was isolated from all GAS-positive cultures using the Gentra Puregene Yeast/Bact. Kit (Qiagen). Purity of DNA was determined by OD_{260}/OD_{280} ratios between 1.8 and 1.9. The DNA concentration of all samples was adjusted to 50 ng/μl. Sequencing of the GAS *emm* gene was performed according to Beall et al. and the advices of the US Center for Disease control and Prevention (CDC) [25, 33]. For technical details,

please refer to supplementary Figure 2. *Emm* types and *emm* clusters were determined according to Sanderson-Smith et al. [26]. The first 250 DNA bases of each *emm* sequence were tested for homology with all *emm* types described in the CDC database [34]. A corresponding *emm* type and/or *emm* subtype was assumed to be valid upon confirmation of at least 97% of the 180 features listed in the CDC *emm* type identification. When variances were above 3%, the respective sequences were re-analyzed for potential alignment with any known *emm* type in the CDC database. For this, we used the software *emm*-blast. Finally, unedited sequence traces were sent to the CDC and all newly identified *emm* subtypes were added to the CDC *emm* sequence database.

Statistical analysis

Patient clinical data and *emm* sequences of all GAS isolates were entered into a study database using MS Excel 2013. To investigate the longitudinal development of *emm* types and *emm* clusters, irrespective of the specific disease or infection site, distributions were compared between the early period (April 1, 2006–March 31, 2007) and the late study period (May 1, 2011–April 30, 2012). All contingency tables were analyzed using Fisher's exact test (SPSS software version 22). Comparisons with many coupled degrees of freedom were performed using the Monte Carlo method. Statistical significance was obtained at a level of $\alpha = 0.05$. In cases where there was multiple testing, results were adjusted by the Bonferroni post hoc test (p_{adj}).

Results

Frequencies of non-invasive GAS infections

During the study period, 683 GAS-positive microbiological cultures were detected. Eight patients presented with two episodes of acute tonsillopharyngitis within 3 months, with the same GAS *emm* type being isolated. These cases were diagnosed as acute recurrent tonsillopharyngitis and included as a single case per patient. In 67 cases, no patient clinical data were available. In 42 additional cases, no positive GAS growth was able to be obtained from the GAS samples following storage at the end of the study period. As shown in supplementary Figure 1, the final study cohort is comprised of 566 cases: 405 cases of tonsillopharyngitis (71.6%), of which 75 cases were diagnosed as scarlet fever (13.3%); 34 cases of skin infection (6.0%); 21 cases of otitis media (3.7%); 19 cases of perineal dermatitis (3.4%); and two urinary tract infection cases (0.4%). In 77 cases, evaluation of the clinical patient data revealed presumable GAS carriers (13.6%). Additionally, eight cases with invasive infections (1.4%) were detected.

Distribution of GAS *emm* types and *emm* clusters

In the study cohort, *emm*12 was the most common *emm* type found (107 cases or 18.9%), followed by *emm*1 (81 cases or 14.3%), *emm*4 (80 cases or 14.1%), and *emm*28 and *emm*89 (with 64 cases or 11.3% each). In considering *emm* clusters, E4 was the most frequent (176 cases or 31.1%), followed by *emm* cluster A-C4 (107 cases or 18.9%), A-C3 (81 cases or 14.3%), and E1 (80 cases or 14.1%). Among the 405 cases with GAS-positive tonsillopharyngitis, *emm*12 was the most common and accounted for 79 cases (19.5%), followed by *emm*4 (62 cases or 15.3%), *emm*1 (56 cases or 13.8%), *emm*89 (54 cases or 13.3%), *emm*28 (37 cases or 9.1%), and *emm*3 (36 cases or 8.9%). With respect to *emm* clusters, the *emm* cluster E4 most frequently was found (120 cases or 29.6%), followed by *emm* cluster A-C4 (79 cases or 19.5%), E1 (62 cases or 15.3%), and A-C3 (56 cases or 13.8%). Among the eight invasive GAS isolates, four were genotype *emm12* and one isolate each was genotypes *emm*1, *emm*4, *emm*6, and *emm*28. *Emm* clusters of the invasive GAS isolates were A-C4 (50.0%), along with clusters A-C3, E1, E4, M6, respectively. An overview of the *emm* types and *emm* clusters is shown in Fig. 1.

Identification of new *emm* subtypes

Within the overall study cohort, the *emm* sequence analysis of three GAS isolates uncovered new, previously unidentified *emm* subtypes. These subtypes were defined as *emm*29.13 (tonsillopharyngeal colonizer), *emm*36.7 (tonsillopharyngitis case), and *emm*75.5 (tonsillopharyngitis case), and subsequently were added to the CDC database. DNA sequences of the new *emm* subtypes are shown in supplementary Figure 3.

Longitudinal development of *emm* types and *emm* clusters

Comparison of GAS isolated during the early study period (01.04.2006-31.03.2007) and the late study period (01.05.2011–30.04.2012) revealed no changes in the epidemiology of single *emm* types. (Fisher's exact test for total and *emm* type-specific distributions: $p(\text{total}) = 0.005$; $p(emm4) = 0.027$, $p(emm4)_{adj} = 0.107$) or *emm* clusters (Fisher's exact test for total and *emm* cluster specific distributions: $p(\text{total}) = 0.004$; $p(\text{cluster E1}) = 0.027$, $p(\text{cluster E1})_{adj} = 0.080$; Fig. 2). Absolute numbers of GAS isolates in the early ($n = 74$) and late ($n = 73$) periods were similar.

Emm types and clusters in tonsillopharyngitis and scarlet fever

The *emm* type and *emm* cluster distribution of GAS isolates derived from patients with tonsillopharyngitis and scarlet fever differed from those of patients with tonsillopharyngitis

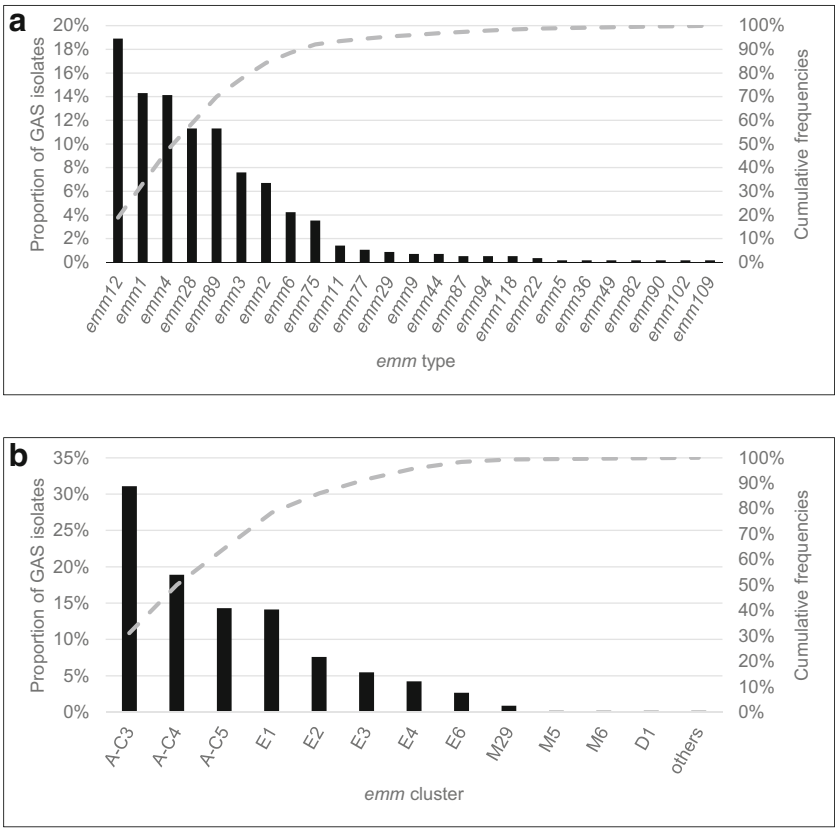

Fig. 1 *Emm* type and *emm* cluster distributions of GAS isolates. The bars show the total frequencies of different **a** *emm* types and **b** *emm* clusters found in the study cohort of $n = 566$ GAS isolates. The lines indicate the cumulative frequencies of *emm* types and *emm* clusters, respectively

and no signs of scarlet fever ($p = 0.000119$ and $p = 0.000005$ by Fishers Exact test via Monte Carlo method). Accordingly, single contingency table analysis revealed *emm4* ($p = 0.00014$; $p_{adj} = 0.0007$) and *emm3* ($p = 0.011$; $p_{adj} = 0.056$), as well as *emm* clusters A-C5 ($p = 0.014$; $p_{adj} = 0.041$) and E1 ($p = 0.00014$; $p_{adj} = 0.00042$), as being more common among patients with tonsillopharyngitis and scarlet fever. In cases of tonsillopharyngitis without scarlet fever, *emm28* ($p = 0.00065$; $p_{adj} = 0.033$), *emm89* ($p = 0.023$; $p_{adj} = 0.11684$), along with *emm* cluster E4 ($p = 0.000036$; $p_{adj} = 0.00011$), were more prevalent. *Emm* type and *emm* cluster distributions in patients with GAS tonsillopharyngitis with and without scarlet fever are shown in Fig. 3.

Emm types and *emm* clusters in perineal dermatitis

As compared with patients with tonsillopharyngitis, the distributions of GAS *emm* types and *emm* clusters in patients with perineal dermatitis displayed a different pattern ($p = 0.000035$ and $p = 0.005$). Single analysis showed *emm28* ($p = 0.000000490$; $p_{adj} = 0.000002$) and cluster E4 ($p = 0.000021$; $p_{adj} = 0.000084$) to be more common in the group of perineal dermatitis. By contrast, *emm12* or cluster A-C4 generally was more frequent in patients with GAS tonsillopharyngitis ($p = 0.031$; $p_{adj} = 0.126$ each). Additionally, subgroup analysis revealed that the distribution of *emm* types and *emm* clusters in patients with perineal dermatitis differed from those in

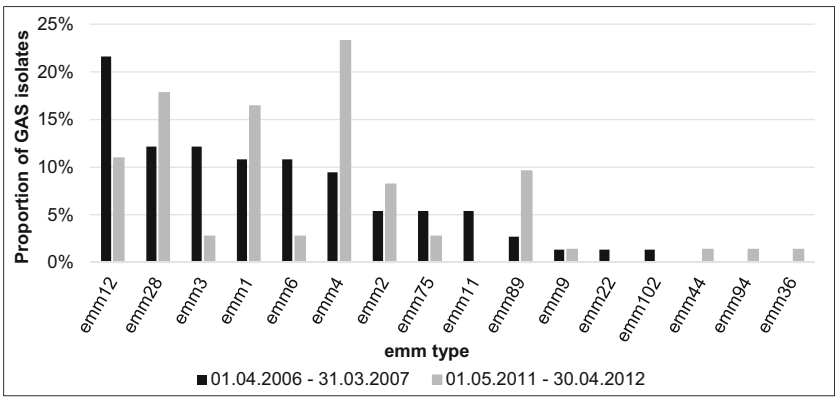

Fig. 2 Temporal distribution of *emm* types. The bar graph shows the proportion of GAS *emm* types during the early period (01.04.2006–31.03.2007; black) and the late period (01.05.2011–30.04.2012; gray). Each period is represented by a total of 74 and 73 GAS isolates, respectively. The bars are shown in decreasing order of the *emm* detection rate during the early study period. No significant temporal changes were detected ($p \geq 0.05$)

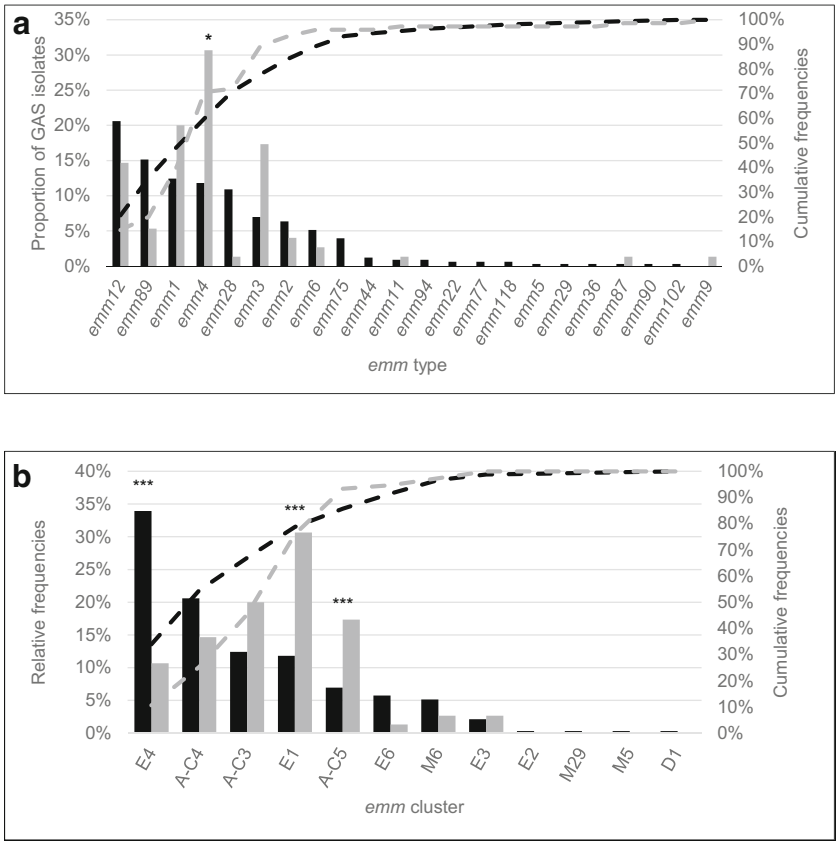

Fig. 3 *Emm* type and *emm* cluster distribution in patients with GAS tonsillopharyngitis with (gray bars or lines) or without (black bars or lines) scarlet fever. The bar graphs show the proportional distribution of different **a** *emm* types and **b** *emm* clusters of GAS isolated from patients with tonsillopharyngitis and no additional symptoms (group 1, black; $n = 330$) and from patients with tonsillopharyngitis and scarlet fever (group 2, gray; $n = 75$). The *emm* types and *emm* clusters are shown in decreasing order of detection in group 1. Cumulative frequencies are presented by the black and gray lines. Besides e*mm4* ($p_{adj} = 0.0007$), *emm* clusters A-C5 ($p_{adj} = 0.041$) and E1 ($p_{adj} = 0.00042$) were more common in patients with GAS tonsillopharyngitis and scarlet fever. Cluster *emm* E4 ($p_{adj} = 0.00011$) was more frequent in patients with GAS tonsillopharyngitis without scarlet fever. Statistical differences by Fisher's exact tests and Bonferroni post hoc tests are indicated as follows: $*p_{adj} < 0.05$; $***p_{adj} < 0.001$

patients with tonsillopharyngitis without scarlet fever ($p = 0.000203$ and $p = 0.022$). In patients with perineal dermatitis, *emm*28 was more frequent ($p = 0.000003$; $p_{adj} = 0.000012$), as was cluster E4 ($p = 0.00013$; $p_{adj} = 0.000254$). In patients with GAS tonsillopharyngitis and no scarlet fever, *emm12* ($p = 0.031484$; $p_{adj} = 0.126$) and cluster A-C4 ($p = 0.031484$; $p_{adj} = 0.0630$) generally appeared more frequently. Additional differences were detected between GAS isolates of patients with perineal dermatitis and GAS isolates of patients with tonsillopharyngitis and scarlet fever ($p(emm\ types) = 1.49 \times 10^{-10}$; $p(emm\ clusters) = 3.14 \times 10^{-8}$). *Emm*28 ($p = 1.20 \times 10^{-8}$; $p_{adj} = 0.000012$) and cluster E4 ($p = 1.36 \times 10^{-8}$; $p_{adj} = 4.09 \times 10^{-8}$) more frequently were found in patients with perineal dermatitis than in patients with tonsillopharyngitis and scarlet fever. On the other hand, *emm*4 ($p = 0.005225$; $p_{adj} = 0.0209$) and cluster E1 ($p = 0.005225$; $p_{adj} = 0.01568$) were more common in patients with tonsillopharyngitis and scarlet fever than they were in patients with perineal dermatitis. *Emm* type and *emm* cluster distributions in the GAS of patients with perineal dermatitis are displayed in Fig. 4.

Discussion

With a total of 566 GAS isolates and an observation period lasting over 6 years, the present study provides, to our knowledge, the largest monocentric epidemiological report on non-invasive GAS *emm* types and associated clinical data in pediatric patients in Germany thus far. The proportion of invasive to non-invasive GAS disease was 1 to 60. Of all GAS-positive throat cultures taken, the proportion of presumable GAS carriers identified during routine patient diagnostics was one in five, as compared with patients with GAS tonsillopharyngitis. Scarlet fever was observed in one out of four patients with GAS tonsillopharyngitis. One out of 50 patients with GAS tonsillop haryngitis experienced a recurrent episode after antibiotic treatment. This data provide representational proportions of different GAS disease manifestations at a tertiary care children's hospital. Our study thereby underlines the additional value of clinical data assessment when combined with microbiological result analysis.

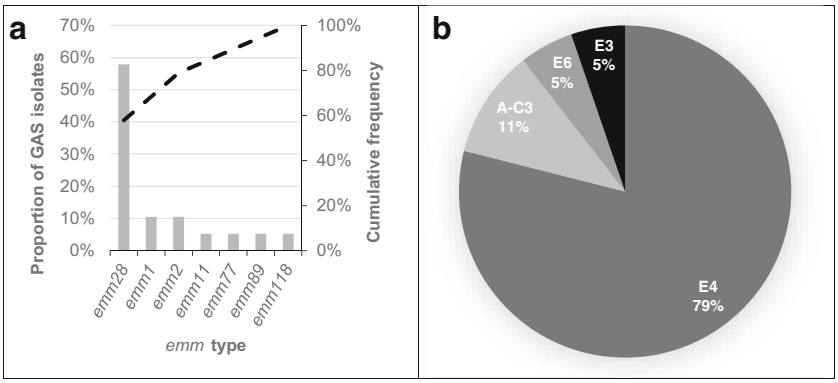

Fig. 4 *Emm* type and *emm* cluster distribution in patients with GAS perineal dermatitis. **a** The bars represent the proportions of different GAS *emm* types. The cumulative frequency of *emm* types is shown as dashed line. **b** Proportions of e*mm* clusters are presented in the pie chart (**a**, **b**: $n = 19$ GAS isolates)

As compared with the Strep-EURO results (2003–2004, [14]), GAS genotyping revealed *emm*12 to be the most common GAS strain in our study (2006–2012). *Emm81* and emm77, the most prevalent isolates in local GAS infection in the Strep-EURO study [14], either were not (*emm81*) or else only very rarely (*emm*77; 1%) were detected in our cohort. Of note, we identified three new *emm* subtypes in non-invasive GAS isolates. These were added to the CDC database. Their sequences are provided in the supplementary material. In line with numerous other reports [1–3, 9], these study results demonstrate the multi-facetedness of GAS and emphasize the need for study updates that can characterize GAS with respect to any potential genomic and epidemiological changes.

In numerous reports on GAS outbreaks, *emm1* and *emm89* strains have been associated with particularly virulent GAS clones [9, 14, 16, 29–31]. Interestingly, however, in our study, *emm1* and *emm89* commonly were found in non-invasive GAS isolates where no outbreaks had been recorded in the service area of the tertiary hospital during the 6-year study period. Together with *emm4*, our study even showed *emm1* to be the second most common genotype among all non-invasive GAS isolates—a finding that significantly differs from the results of studies from Spain (1998–2009) and France (2009–2011), both of which found *emm1* to be less common among non-invasive GAS infections [17, 35]. In accordance with the French study, where *emm4* and *emm89* more frequently were found in children with tonsillopharyngitis than in patients with invasive GAS diseases [35], our study confirmed *emm*4 and *emm*89 to be among the most prevalent *emm* types in non-invasive GAS isolates. As described for the invasive GAS found in our study, *emm12* also was the most common genotype in non-invasive GAS isolates; no major differences between invasive and non-invasive GAS *emm* types and *emm* clusters were observed. It is possible that this is due to the relatively small number of invasive GAS isolates derived from the local patient cohort. However, the local burden of GAS diseases was stable and no major changes of single *emm* types or *emm* clusters were found during the 6-year study period. This suggests our study results may be seen as consistent and representative.

With regard to GAS disease manifestations, *emm*4, along with cluster A-C5 and cluster E1, together were more common in patients with tonsillopharyngitis and scarlet fever than they were in patients with tonsillopharyngitis without scarlet fever. This finding reaffirms supports for the observation of GAS *emm*4 accumulation in scarlet fever patients [35]. For GAS isolated from skin infections as compared with tonsillopharyngitis isolates regardless of scarlet fever, no significant differences in the *emm* type distributions were found. The only exception to this rule was cluster E4, which was more common in GAS of patients with skin infections than it was in the subgroup of tonsillopharyngitis patients with scarlet fever. In perineal dermatitis, GAS isolates of cluster E4 also were frequent (80%) and, together with *emm*28 (58%), indicated a narrow spectrum of perineal GAS *emm* types and *emm* clusters. The predominance of GAS cluster E4 and *emm*28 in perineal dermatitis was additionally supported by the finding that *emm*28 and cluster E4 were more frequent in these GAS isolates than they were in patients with tonsillopharyngitis. The different *emm* types of GAS isolates derived from patients with tonsillopharyngitis and perineal dermatitis may indicate oropharyngeal-to-perineal transmission of GAS infections to be rare among the population represented by our study cohort. The majority of *emm*28 in GAS isolates of patients with perineal dermatitis additionally was documented in a study on perineal infections in a pediatric practice [36].

Regarding vaccine coverage, 98.0% of all study patients with GAS infections would have been covered by the 30-valent M protein-based GAS vaccine [37]—a vaccine that includes coverage of 100% invasive and perineal GAS isolates, as well as coverage of 100% GAS isolates of patients with otitis media, 98.3% with tonsillopharyngitis and 91.2% with pyoderma.

On the other hand, one also could speculate that the M-based immunity derived from the prevalent non-invasive GAS infections with *emm*1 and *emm*89 strains described by the current study could have been a factor protecting against outbreaks of invasive *emm1* and *emm89* infections in the study region. Then again, the absence of outbreaks also could

reflect a multifactorial decrease in susceptibility of the study population to these *emm* types. Alternately, it could indicate less virulent *emm1* and *emm89* clones circulating in the study region. Therefore, it could be interesting to compare *emm1* and *emm89* GAS isolates of this cohort to other invasive *emm1* and *emm89* strains. Nonetheless, the predominant role of *emm12* in the invasive as well as non-invasive study isolates strongly supports the hypothesis that invasive diseases caused by certain GAS clones reflect a widespread transmission of these clones in the respective population, rather than their virulent potential per se [32].

In conclusion, this study provides a comprehensive characterization of a large, local GAS cohort of pediatric patients seeking treatment at a tertiary medical center. Although the transferability of *emm* type and *emm* cluster distributions to other study populations may be limited, there are considerable advantages to a monocentric study design. As described in relation to other multicenter studies, this study compares and contrasts GAS genotypes and disease manifestations for one particular, local cohort over a 6-year period, thereby minimizing regional variation. Our study documents considerable deviations from previous epidemiological findings, especially with respect to the frequent detection of *emm1* and *emm89* in non-invasive GAS infections. These observations suggest that continuous monitoring of molecular and epidemiological GAS characteristics is needed in order to track the multifaceted pathogenic potential of GAS.

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Data availability Data are available upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethic approval All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study, formal consent is not required.

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