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Genetic evolution of invasive *emm28 Streptococcus pyogenes* strains and significant association with puerperal infections in young women in Finland

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Abstract

Objectives: *Streptococcus pyogenes* or group A streptococcus (GAS) is a human specific pathogen that annually infects over 700 million individuals. GAS strains of type *emm28* are an abundant cause of invasive infections in Europe and North America.

Methods: We conducted a population-based study on bacteraemic *emm28* GAS cases in Finland, from 1995 to 2015. Whole-genome sequencing (WGS) was used to genetically characterize the bacterial isolates. Bayesian analysis of the population structure was used to define genetic clades. Register-linkage analysis was performed to test for association of *emm28* GAS with delivery- or postpartum-related infections. A genome-wide association study was used to search for DNA sequences associated with delivery or puerperal infections.

Results: Among 3060 bacteraemic cases reported during the study period, 714 were caused by *emm28*. Women comprised a majority of cases (59 %, 422/714), and were significantly over-represented (84.4 %, 162/192, $p < 0.0001$) among cases in the childbearing age group (20–40 years). Register-linkage analysis revealed strong association ($p < 0.0001$) of *emm28* bacteraemias

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Author contributions

J.V., J.M.M. and K.G.Y.H. designed the study. K.G.Y.H., S.B.B. and T.K. analysed the data. H.L.H. contributed in the data acquisition. K.G.Y.H. and J.V. wrote the manuscript and produces the figures and tables. All authors gave feedback and participated in the manuscript editing.

Transparency declaration

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Appendix A. Supplementary data

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with delivery and puerperium. In this register-linkage analysis, 120 women with GAS bacteraemia were identified and linked to delivery, infections during delivery or puerperium time. Among these the proportion of cases caused by e*mm28* was significantly higher than any other *emm* type (55.8%, 67/120, $p < 0.0001$). Among the four genetic subclades identified, SC1B has dominated among the bacteraemic cases since 2000. Altogether 620 of 653 (94.9%) isolates belonged to SC1B. No specific sequence or genetic clade was found nonrandomly associated with delivery or puerperal infections.

Conclusions: Women of childbearing age were significantly overrepresented among bacteraemic *emm28* GAS cases, and in particular were strongly associated with delivery and puerperium cases over the 21 years studied. The molecular mechanisms behind these associations are unclear and warrant further investigation.

Keywords

Bacteraemic; Group A streptococcus; Puerperal sepsis; *Streptococcus pyogenes*; Whole-genome sequencing

Introduction

Streptococcus pyogenes or group A streptococcus (GAS), is a strict human pathogen capable of causing a wide spectrum of infections from mild pharyngitis to severe life-threating conditions such as bacteraemia. Global morbidity and mortality caused by GAS infections is substantial, causing over 700 million infections and over 500 thousand deaths annually [1]. Although far less frequent than mild superficial infections, mortality for invasive GAS infections such as bacteraemia can be very high [2].

The major surface antigen produced by GAS is the M protein, encoded by the *emm* gene. GAS strains are classified into *emm* types and subtypes, based on DNA sequence variation in the variable region of the *emm* gene. At present more than 240 *emm* types have been recognized. The distribution of GAS *emm* types causing infections fluctuates geographically and temporally [3]. Population-based whole-genome sequencing (WGS) studies on GAS isolates have revealed important molecular genetic evolution in the bacterial genome that have increased the pathogenicity and enhanced the spread of newly emergent GAS clones [4,5].

In Finland, the overall incidence of invasive GAS cases has steadily increased since 1995. Importantly the incidence of invasive GAS infections has increased from 2.22 cases per 100,000 inhabitants in 2000 to 3.26/100,000 inhabitants in 2015 [6].

Emm28 strains have consistently remained among the three most prevalent *emm* types causing invasive GAS infections in Finland since 2003 [7–9]. Other numerically dominant *emm* types in invasive GAS infections since 2008 have been *emm1* and *emm89* [8]. A predominance of *emm28* invasive GAS infections has also been observed in several other countries, including other Nordic countries, namely Sweden, Norway and Iceland [7,10–14]. For mainly unknown molecular reasons, *emm28* GAS strains are frequently associated with infections in young women of childbearing age, especially in maternal postpartum infections

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encountered after delivery [7,15–17]. Previously, it was reported that in the age group of 30–39 years invasive GAS *emm28* infections were strongly associated with women (80%, $p < 0.001$) [7]. In Finland the fertility rate for women in the 20- to 40-year age group is substantially higher than for younger and older age groups [18].

Recently, we performed a comprehensive WGS-based population genomic analysis of a large cohort of 2101 invasive *emm28* isolates collected from six countries, including Finland [19]. The primary goal of the current study was to further analyse the Finnish isolates by investigating the population genomic data in conjunction with demographic, temporal and spatial information of the cases.

Additionally, we searched for pregnancy- or delivery-linked clinical information among bacteraemic *emm28* cases and analysed it in relation to the population genomic structure. Here we report a comprehensive, population-based study of 714 GAS *emm28* cases covering 21 years.

Methods

Bacterial isolates

Since 1995, all clinical microbiological laboratories from Finland are required to report each GAS isolate cultured from blood and/or cerebrospinal fluid to the National Infectious Disease Register (NIDR) maintained by the Finnish institute for health and welfare (THL) [9].

Clinical microbiological laboratories submit the corresponding GAS isolate to the National Reference Laboratory (NRL). All isolates sent to the NRL are emm typed according to the guidelines of the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA; http://www.cdc.gov/streplab/protocol-emm-type.html). *Emm* type has been assigned for all bacteraemic GAS cases since 2003. Before that, T-typing was used to serotype GAS isolates. Isolates assigned as T28 were *emm* sequenced to determine the *emm* type [20]. In this study, all 714 bacteremic GAS *emm28* strains isolated between 1995 and 2015 from the NRL strain collection were investigated.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed for 684 *emm28* GAS isolates. AST was performed using the disc diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (version 5.0). Antimicrobial susceptibility was tested against erythromycin, clindamycin, tetracycline and levofloxacin.

Data analysis

Annual incidence rates for bacteraemic *emm28* cases were calculated using population data obtained from Statistic Finland, a public authority, which informs the national statistical service (https://www.stat.fi/index_en.html). To analyse for association of bacteraemic GAS isolates with infections related to delivery or puerperium, we performed a register-linkage study between NIDR and the Hospital Discharge Register maintained by THL. Included

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were all 267 bacteraemic GAS cases in women aged 20–40 years during years 2004–2015. For these cases, International Classification of Diseases v10 (ICD-10) codes related to delivery, or infections related to delivery or puerperium or GAS (Supplementary Table S1) within a period of 30 days before to 30 days after the isolation of a bacteremic GAS were searched for from the Hospital Discharge Register.

Chi-squared test (Prism 4, GraphPad Software) was used to compare the proportions of women with delivery or infections related to puerperium to those without. The difference was considered significant if the p-value was <0.05.

WGS and genome wide association study

DNA extraction, library preparation and WGS using an Illumina NextSeq550 instrument were performed as described previously [4]. Genome analysis was performed as described previously [19]. In brief (see Supplementary Material for detailed methods for WGS and genome-wide association study (GWAS)), after removal of artifacts and low-quality bases, base call corrected reads were aligned to *emm28* reference genome MGAS6180. Single nucleotide polymorphisms (SNPs) residing in the core chromosome after correction for recombination were used to infer phylogeny and to cluster genetically related strains, as described previously [19].

Ethics

The study was conducted in accordance with the Declaration of Helsinki and national and institutional standards. Ethical committee clearance was not needed for this retrospective register-based study. Permission for this study was obtained from the Finnish Institute for Health and Welfare (THL/1121/5.05.00/2017).

Results

Invasive GAS cases

Altogether 3060 bacteraemic GAS cases were reported to NIDR from January 1995 to December 2015. Of these, 714 (714/3060, 23.3%) cases with available corresponding strains were caused by type *emm28*. The yearly proportions and the corresponding incidences of *emm28* cases among all bacteraemic GAS cases are shown in Fig. 1. The yearly proportion of *emm28* GAS cases varied between 8.8% (seven out of 80 GAS bacteraemias) in 1997 and 46.2% (55/119) in 2003.

The median age of *emm28* bacteraemic GAS cases was 54 years (range, 13 days to 94 years). Women accounted for 59% of cases (422 women from altogether 714 GAS cases), men 40% (283/714), and for 1% (9/714) gender data were not available. To investigate for an association of *emm28* infections with women of childbearing age, we divided the cohort into four age groups (<20, 20–40, 41–60 and > 60 years). Women were significantly overrepresented ($p < 0.0001$) in the 20- to 40-year childbearing age group. From altogether 192 cases in this age group, 162 were women, which comprises 84.4% of the cases. In all other age groups combined ($n = 512$), the distribution of cases among men and women was relatively equal, altogether 252 men (252/512, 49 %) and 260 women (260/512, 51 %) (Fig.

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1(c)). The same comparison was done with all non-*emm28* bacteraemic GAS infections in Finland for years 2004–2015. No similar overabundance was observed (Fig. 1(c)).

Antimicrobial susceptibility testing was performed for altogether 684 *emm28* GAS isolates. Resistance against all tested antibiotics remained low; erythromycin 1.9 % (*n* = 13), clindamycin 0.9 % (*n* = 6), tetracycline 0.7 % (*n* = 5) and levofloxacin 1.3 % (*n* = 9) of resistance, respectively.

Register-based linkage analysis

To explore the nature of the overabundance of bacteraemic *emm28* infections among women of childbearing age, a register-based linkage analysis was performed. Specific ICD-10 codes linked to deliveries, infection related to delivery or puerperium, temporally linked to the bacteraemic GAS infection were searched (Supplementary Table S1). Altogether 267 cases were included in the analysis. These cases were caused by 30 different *emm* types (Table 1). *Emm28* was the most frequently observed *emm* type, representing 42.3% (*n* = 113) of the cases. Of the 267 cases, 112 had an ICD-10 code indicative of a delivery and from these, in 63 cases (58%) the infection was caused by *emm28* ($p < 0.0001$, Table 1). Among cases with ICD-10 codes for puerperal sepsis or infections related to delivery or puerperium time, *emm28* was the most frequently observed *emm* type (55.3%, 52/94, $p = 0.0015$, Table 1). Searching for any of the ICD-10 codes related to delivery, infections during delivery or puerperium time, identified 120 cases, and among these the proportion caused by e*mm28* was highly significant (55.8%, 67/120, $p < 0.0001$, Table 1).

WGS analysis

Genomic analysis was performed for all available Finnish bacteraemic *emm28* GAS isolates (*n* = 714; Supplementary Table S2) from 1995 to 2015. Of these, 704 were also part of the large study of bacteraemic *emm28* GAS isolates from six countries [19]. The Finnish strains were sequenced to a mean depth of 207-fold coverage (range 34- to 815-fold). To analyse the genome-wide distribution of single nucleotide polymorphisms (SNPs) and genetic relationships among the strains, core chromosomal SNPs were used. Two isolates were very distant genetic outliers and were excluded from further analysis. Among the 712 remaining isolates, in the aggregate, there were 5110 SNPs identified in the recombination-filtered core genome (that is, with SNPs acquired by horizontal gene transfer removed from the core genome). Bayesian analysis of population structure (BAPS) hierarchically clustered the strains in two major primary clades (1 and 2), and into four secondary subclades (designated SC1A, SC1B, SC2A and SC2B) [19]. A majority of the 712 Finnish strains are part of Clade 1 (*n* 699) of which the majority are part of SC1B (*n* = 628) (Fig. 2(a)). Only a minor proportion of the Finnish strains belongs to Clade 2 (*n* = 13). From the temporal data of isolates, it was discovered that SC1B started to predominate after year 2000 by replacing SC1A entirely (Fig. 2(a)).

Multilocus sequence typing (MLST) types of the strains were determined from the WGS data. Four sequence types (STs) were discovered, ST52, ST456, ST45 and ST244, respectively (Fig. 2(b)). ST52 and ST456 predominated covering 82.4 % (*n* = 587) of the strains.

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With our comprehensive population-based collection covering 21 years, we were able to study temporal dynamics of the population in relation to the phylogenetic tree. There was a clear temporal correlation observed with inferred phylogeny (Fig. 2(c)). A clear shift in the phylogeny is seen with the change from SC1A to SC1B in the early 2000s. Although there is a significant non-random association between GAS infections in women of childbearing age and *emm28*, strains isolated from women in the 20- to 40-year age group were found randomly distributed relative to the *emm28* isolate phylogenetic tree (Fig. 3).

We performed a further GWAS analysis on 136 *de novo* assembled *emm28* genomes. Using this approach, no genetic content of the isolates was found significant associated with infections related to delivery or puerperium (data not shown).

Discussion

In this study we showed that in Finland, young women of childbearing age are significantly over-represented in bacteraemic *S. pyogenes* cases caused by *emm28*. With further register-linkage analysis, we observed a clear association of *emm28* GAS bacteraemias with deliveries and puerperal infections in young women. In addition, with WGS data, we show that the genome of *emm28* GAS has evolved during the 21 years studied.

S. pyogenes is an important human pathogen with a wide disease spectrum and global dissemination. The resurgence of invasive GAS cases has been reported in several countries [2,21]. In the early 2000s, increased incidence rates were reported especially due *emm1* and *emm89* [5,8,10,22,23]. In Finland, despite epidemic waves caused by *emm1* and *emm89*, the predominant *emm* type causing invasive GAS infections since 2004 has been *emm28* [8,24]. Our study consists of a genome-wide analysis of bacteraemic *emm28* GAS isolates collected during 21 years and an in-depth analysis of invasive infections in young women of childbearing age.

Emm28 accounted almost one-fourth (23.3 %) of all invasive GAS cases reported during the study period. Previous epidemiological studies on invasive GAS cases in Finland for years 2004–2013 [8,24] found *emm28* GAS as the most prevalent *emm* type. Similar to Finland, several other countries report e*mm28* GAS as one of the most common types in invasive infections [10,11].

Several predisposing factors have been described for invasive GAS infections. These include age extremes, ethnicity, underlying conditions such as heart disease [25]. In general, male gender is considered as a risk factor for invasive GAS infection [2,25]. On the contrary, in women, puerperal sepsis is a rather rare but severe disease condition, and GAS is one of the well-recognized causatives for it [16,17,26]. In a large study conducted in the USA, the incidence of pregnancy- and postpartum-related severe GAS infections were 0.2 and 0.55/1000 woman-years, respectively [26]. In the Strep-EURO study which included 11 European countries, in 4% of invasive GAS cases the disease manifestation was puerperal sepsis [7]. Similarly, in a Finnish study covering 10 years of bacteraemic GAS cases in one Health district, in 8% of the cases the presenting clinical manifestation was puerperal sepsis [27]. For postpartum women, the risk for infection caused by GAS is 20-fold higher

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compared with non-pregnant women [26]. In our cohort, *emm28* GAS was clearly associated with delivery and infections related to delivery or puerperium (Table 1). Our result is supported by previous studies on the association of *emm28* GAS with invasive postpartum infections [15,16,26].

In the WGS analysis of the isolates, SC1B was discovered as the predominant *emm28* genetic lineage in Finland. The proportion of SC1B has increased also in other countries including the USA, Iceland and Norway [19]. It remains uncertain whether the displacement of SC1A with SC1B was a random effect or happened due to the effect a specific SNPs.

Previous studies on *emm89* and *emm1* have shown that acquisition of genetic material with horizontal gene transfer (HGT) has resulted in the increased production of secreted toxins and led to a more virulent phenotype of the bacteria [4,28]. A similar type of HGT event was discovered in *emm28* GAS isolates in Clade 2, which resulted in the acquisition of 28.0-kilobase genetic block from *Streptococcus dysgalactiae* subspecies *equisimilis* that encodes secreted toxins NAD + -glycohydrolase (SPN) and streptolysin O [19]. The frequency of Clade 2 increased in the USA from early 2000 but not in other countries included in that study. Only a scarce proportion of the Finnish isolates were part of Clade 2 and all were isolated before 2000 (Fig. 2(a)).

We did not find any subset of the *emm28* isolates studied or differing locations in the genome that were non-randomly associated with cases related to puerperal sepsis or delivery. This finding suggests that there is no distinct clone of the *emm28* isolates studied, which would cause these specific infections.

The strength of our study is clearly the nationwide nature of the collection which includes all bacteraemic *emm28* GAS cases from Finland. Our study emphasizes the observation that delivery- and postpartum-linked infections caused by GAS are a noteworthy clinical manifestation in Finland, and *emm2*8 has a significant role in these. With our comprehensive population-based collection covering 21 years, we were able to study temporal dynamics of the population in relation to the phylogenetic tree. There are certain limitations in this study as well. Our search for clinical risk factor data in young women with GAS bacteraemia was limited only to certain disease manifestations recorded by a limited number of ICD-codes. A different type of study design might identify more cases increasing the power of the analysis. The number of strains included in the GWAS analysis was limited and it might affect the power of the analysis.

There are several factors that might contribute to the postpartum GAS infections such as altered immune status of the mother, disruption of the tissues, spread of the pathogen from an another infection site such as pharyngitis and through nosocomial exposure [29]. None the less, none of these factors as such explains the high prevalence of *emm28* in the infections. The *emm28* GAS genome contains an integrative conjugative element called region of difference (RD)2, which is thought to be horizontally acquired from group B streptococci (GBS) [30,31]. RD2 encodes several surface proteins, which may play a role in the tissue tropism by attaching to endometrial epithelial cells [32,33]. However, vaginal GAS colonization during pregnancy is known be to very low, less than 1% [34,35], and most

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likely it does not explain the prevalence of postpartum *emm28* GAS infections. Asymptomatic carriage of GAS on skin or throat are more common (5–30% of the population), and community carriage might predispose to postpartum infections [29].

In conclusion, a significant association between bacteraemic *emm28* GAS, young women and infections related to delivery and puerperium was identified. This is a first descriptive study of *emm28* GAS bacteraemias from Finland and the results warrant further studies to identify the possible underlying mechanism behind the association of *emm*28 GAS with maternal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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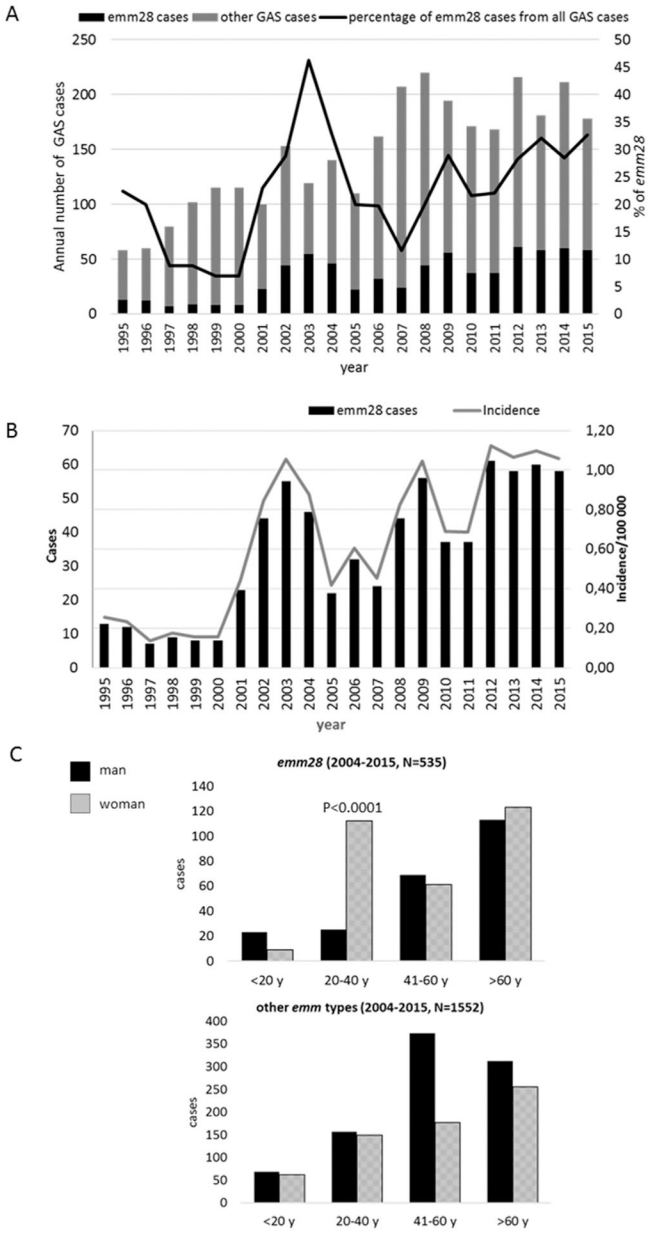

Fig. 1.

(a) Bacteraemic group A streptococcus (GAS) cases reported to the National Infectious Disease Register between 1995 and 2015, and the proportion of *emm28* GAS cases in relation to all cases. *Emm* typing has been used as reference typing method for all GAS isolates since 2003 (*n* total = 3060). (b) Annual number of cases and incidence of bacteraemic *emm28* GAS cases, Finland, 1995–2015 (*n* total = 714). (c) Total number of bacteraemic GAS cases in Finland between 2004 and 2015 by gender and age groups in type *emm28* (upper part) and the rest of the *emm* types (lower part).

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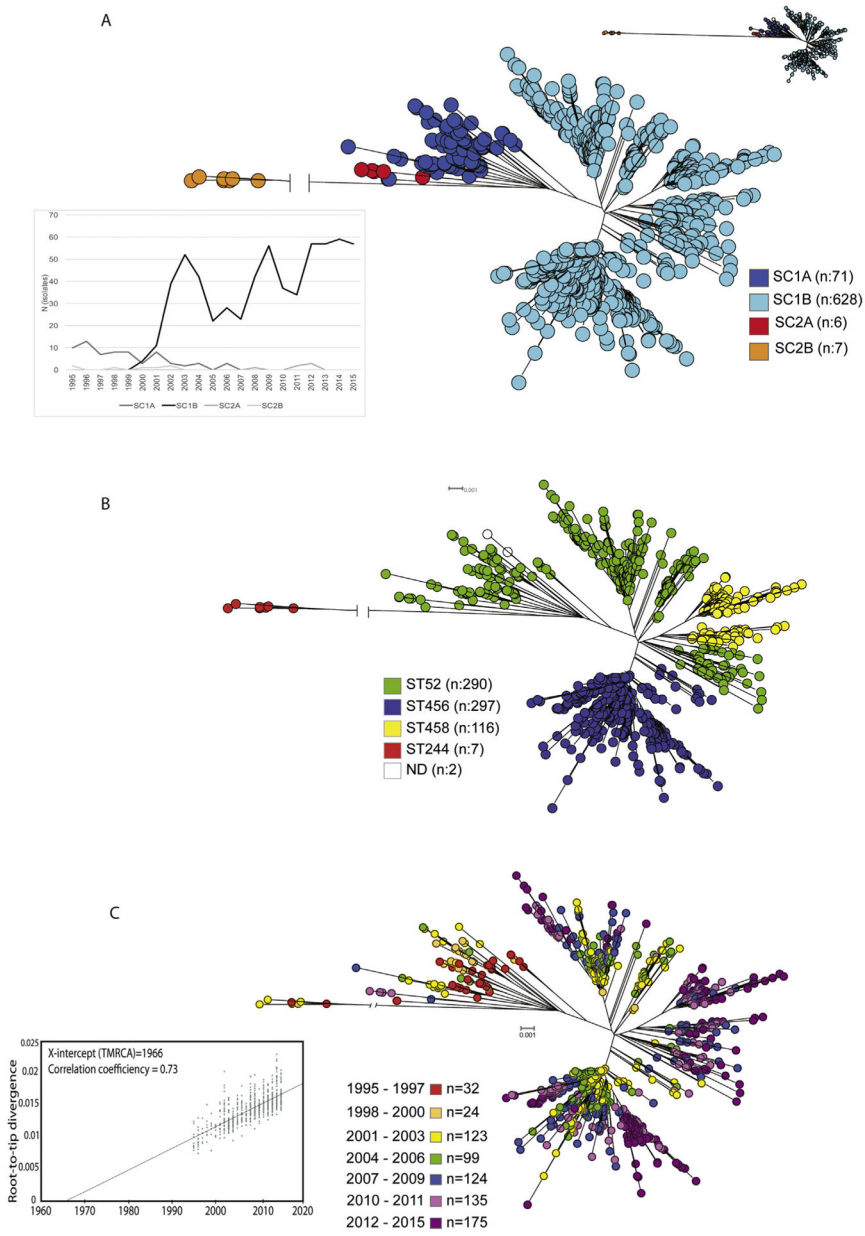

Fig. 2.

(a) Neighbour-joining tree of 712 *emm28* isolates with 5110 core single nucleotide polymorphisms (SNPs). Two primary clades 1 and 2 were further divided to subclades (SC) A and B. Lower left part of the figure: expansion of SC 1B in the early 2000s. (b) Multilocus sequence typing (MLST) distribution in neighbour-joining tree of 712 *emm28* group A streptococcus (GAS) isolates. (c) Temporal association of the 712 *emm28* GAS isolates with isolation year in neighbour-joining tree. Left corner: a root-to-tip genetic distance for 705 Finnish isolates after excluding isolates ($n = 7$) with the region of recombination to constrain the inference to primarily vertically inherited SNPs. The X-intercept is the time of origin of the most recent common ancestor (TMRCA = 1966).

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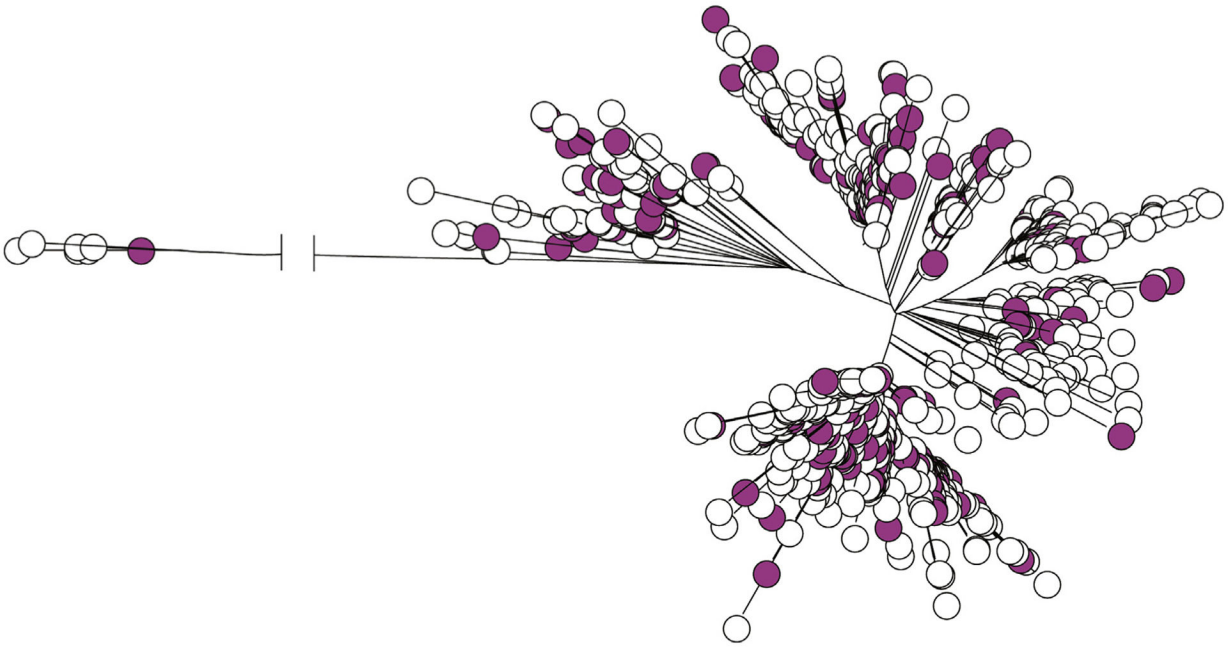

Fig. 3.

Distribution of isolates from women aged 20–40 years (magenta circles, $n = 162$) in the neighbour-joining tree of all 712 group A streptococcus (GAS) isolates included in the study.

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Table 1

Register-linkage analysis was performed for all invasive GAS cases (n = 267) recorded in women aged 20–40 years between 2004 and 2015

emm type	All *n* (%)	Delivery *n (%)*	Infections related to delivery or puerperium, *n* (%)	Delivery or infection related to delivery or puerperium, *n* (%)
emm28	113 (42.3)	63 (56.3) *	52 (55.3) *	67 (55.8) *
emm89	39 (14.6)	13 (11.6)	13 (13.8)	15 (12.5)
emm12	16 (6.0)	5 (4.4)	5 (5.3)	7 (5.8)
emm1	22 (8.2)	9 (8.0)	6 (6.4)	9 (7.5)
emm4	14 (5.2)	9 (8.0)	8 (8.5)	9 (7.5)
emm75	8 (3.0)	2 (1.8)	2 (2.1)	2 (1.7)
other *emm* types **	55 (20.6)	11 (9.8)	8 (8.5)	11 (9.2)
Total	267	112	94	120

The Hospital Discharge Register was used to search for specific ICD-10 codes related to delivery, or infections related to delivery or puerperium (Supplementary Table S1). Table 1 summarizes the distribution of *emm* types in cases for which specific ICD-10 codes were recorded.

* $p < 0.05$.

** other *emm* types (number of cases): *emm119.1* (7), *emm84* (6), *emm27G.6* (4), *emm50* (4), *emm77* (4), *emm118* (3), *emm22* (3), *emm66* (3), *emm25* (2), *emm110.1* (2), *emm73* (2), *emm81* (2), *emm78.3* (2), *emm104* (1), *emm102.3* (1), *emm11* (1), *emm112.2* (1), *emm33* (1), *emm2* (1), *emm60.1* (1), *emm177* (1), *emm87* (1), *emm79.2* (1), *emm8* (1).

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