emm typing of invasive T28 group A streptococci, 1995–2004, Finland

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A total of 985 group A streptococcus (GAS) bacteraemia isolates collected in Finland during 1995–2004 were T-serotyped, and of these, 336 isolates of serotype T28 were subjected to further emm typing. The total number of isolates referred per year showed an increase within the study period, from 43 in 1995 to 130 in 2004. The annual incidence of invasive GAS (iGAS) bacteraemia showed a general increase during the study period, from 1·1 to 2·5 per 100 000 population. Serotype T28 remained among the most common serotypes, in addition to serotypes T1B3264 and T1. The serotype T28 isolates were found to be distributed across six distinct emm types: emm28, emm77, emm53 (including subtypes 53.2 and 53.4), emm87, emm2 and emm4. The serotype distribution and the emm type distribution of serotype T28 fluctuated over time. Within the study period, the proportion of T28/emm28 isolates became the most prominent. During periods of low emm28 incidence, emm types 77 and 53 seemed to show a resurgence. emm typing revealed T28 isolates to be a genetically heterogeneous group harbouring a variety of distinct M proteins. This study confirms that T serotyping alone is not a sufficient method for epidemiological surveillance of iGAS.

INTRODUCTION

Streptococcus pyogenes (group A streptococcus, GAS) causes a variety of diseases ranging in severity from mild respiratory tract infections to invasive infections, including cellulitis, bacteraemia, necrotizing fasciitis and toxic-shock syndrome. Invasive GAS (iGAS) diseases constitute a major global burden, resulting in hundreds of thousands of cases each year, most of which occur in less-developed countries (Carapetis et al., 2005). Population-based surveillance studies, mainly from developed countries, have documented changes in the epidemiology of GAS (Lamagni et al., 2005), and have indicated the importance of type identification for epidemiological studies (Efstratiou, 2000). The availability and application of discriminatory typing methods are essential to these studies.

The classical serological typing schemes for GAS are based on the variability of surface-exposed proteins, such as the T protein, the serum opacity factor (SOF) protein and the M protein (Johnson et al., 1996). The T-agglutination test introduced in 1965 has provided a useful tool for initial screening and characterization of GAS when combined with the SOF reaction result (Moody et al., 1965; Maxted et al., 1973; Johnson & Kaplan, 1993; Johnson et al., 1996). However, the value of the serological typing methods is limited by the specificity and availability of typing sera. Conventional methods are therefore being replaced or augmented by molecular methods.

The major GAS virulence factor, streptococcal M protein, has multiple functions in the pathogenicity of the bacterium, including its contribution to the antiphagocytic capacity of the bacterium, thereby promoting its invasiveness (Fischetti, 1989). M proteins or M-like proteins have been identified also in group C and G streptococci (Collins et al., 1992; Bisno et al., 1996). The serological antigen typing introduced by Rebecca Lancefield provided the basis of GAS M typing, and has been further enhanced by the implementation of molecular approaches directed towards
the M protein emm gene (Lancefield, 1962; Podbielski et al., 1991; Beall et al., 1996; Saunders et al., 1997; Facklam et al., 1999). emm typing is based on the determination of differences in the 5’ end of the emm gene encoding the hypervariable and outward-projecting amino-terminal portion of the M protein (Fischetti, 1989; Beall et al., 1996). The accuracy and unambiguity of emm sequence typing has made it the current ‘gold standard’ method (Efstratiou, 2000), which for the purpose of detecting clonality of strains is most useful when complemented by other molecular typing methods, such as ribotyping, sof sequencing, PFGE, restriction endonuclease analysis (REA) and multilocus sequence typing (MLST) (Single & Martin, 1992; Seppala et al., 1994; Beall et al., 2000; Enright et al., 2001; McGregor et al., 2004; Doktor et al., 2005).

At present, 83 unique M types encoded by unique emm gene sequences (up to type M93) have been validated (Facklam et al., 2002; Johnson et al., 2006). Over 110 emm types (up to type emml124) have also been validated and submitted to the Streptococcus pyogenes emm sequence database at the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). In addition, the database currently contains 47 provisional sequence types (sts) for S. pyogenes (http://www.cdc.gov/ncidod/biotech/strep/emmtypes.htm).

In this study, serotype T28 was the focus of interest because of its dominance among Finnish iGAS isolates. Serotype T28/M28 strains are commonly reported among the top five most common causes of invasive and pharyngitis infections in several countries, in addition to being predominant among cases of puerperal sepsis and neonatal GAS infection (Colman et al., 1993; O’Brien et al., 2002; Tyrrell et al., 2002; Moses et al., 2003; Shulman et al., 2004; Green et al., 2005b; Raymond et al., 2005). In order to have a better understanding of the epidemiology and clonality of Finnish T28 isolates, emm typing was performed for all GAS isolates reacting with anti-T28 typing serum from the study period of 1995–2004.

METHODS

Surveillance of invasive GAS disease. In Finland, iGAS disease, defined as GAS isolated from blood and cerebrospinal fluid, has been notifiable by law since 1995. Clinical microbiology laboratories report (generally electronically) all invasive S. pyogenes isolations to the National Infectious Disease Register (NIDR) at the National Public Health Institute of Finland (KTL) (http://www3.ktl.fi/stat). In addition, the corresponding GAS isolates are referred to the national reference laboratory at KTL for confirmation and typing. In this study, notifications and isolates concerning S. pyogenes blood isolations were included. The isolates were cross-checked to match the corresponding notifications.

Bacterial identification. The referred isolates were confirmed as S. pyogenes by beta-haemolysis on blood agar, sensitivity to bacitracin, and detection of Lancefield group A antigen by latex agglutination (Streptex).

T serotyping. T serotyping was performed using five polyvalent and 21 monovalent anti-T agglutination sera (1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 18, 22, 23, 25, 27, 28, 44, B3264 and Imp19) (Sevac), as described elsewhere (Moody et al., 1965).

emm typing. emm typing was performed jointly at the Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories (RML) (most isolates from 1995 to 1999) and at KTL (mainly isolates from 2000 to 2004). emm typing at RML was performed using primers emml1 and emm2 by previously documented methods (Green et al., 2005a). At KTL, primers MFI (forward), 5’ ATG AGC AGC ATA AAA ATG GCT 3’, and MR1 (reverse), 5’ AGC TTA GTT TTC TTC TTT GCG 3’, were used under the following conditions: initial denaturation at 95 °C for 10 min and 94 °C for 3 min, 35 cycles of denaturation at 93 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 2 min, with a final extension step at 72 °C for 10 min. PCR products were purified with the QiAquick PCR purification kit (Qiagen), as described by the manufacturer. The emm sequencing reaction was performed with primer MFI and BigDye chemistry (Applied Biosystems), as described by the manufacturer, and analysed with an ABI Prism 310 genetic analyser (Applied Biosystems). The sequence data were compared with the CDC Streptococcus pyogenes emm sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). emm types were assigned on the basis of ≥95% sequence identity with the exact 150-base type-specific sequences as specified by the database (http://www.cdc.gov/ncidod/biotech/strep/assigning.htm).

Statistical analysis. Disease rates were calculated using resident population data as the denominators for the corresponding year. Data were analysed using the statistical software Intercooled Stata 9.1 for Windows (StataCorp). Categorical data were compared using the chi square test. Differences were considered significant when P<0.05.

RESULTS AND DISCUSSION

Epidemiology of iGAS disease in Finland

During 1995–2004, 1034 cases of iGAS disease (blood bacteraemia) were reported in Finland, and from these, 985 corresponding isolates were submitted to the reference laboratory at KTL (Fig. 1). The annual incidence of iGAS bacteraemia showed a general increase, from 1·1 to 2·5 per 100 000 population during the period 1995–2004, peaking in 2002 (2·9 per 100 000 population). The annual number of submissions also increased threefold, from 43 to 130. The reasons for this increase are currently undetermined. The overall coverage of the notification system and sending of isolates to KTL in Finland is good and has remained stable over this period. On average, we received an isolate for typing in 95% of the notified cases. The disease rates of GAS bacteraemia in Europe have recently been reported to be between 1·7 and 3·95 per 100 000 population in the early 2000s, varying considerably by country but showing a general increasing trend over the past two decades in most countries (Lamagni et al., 2005). The Finnish trend follows this pattern. In the USA, the disease rate of iGAS (including bacteraemia) has been estimated to be around 3·5 per 100 000 population from the late 1990s onwards (http://www.cdc.gov/ncidod/dbmd/abcs/reports.htm) (O’Brien et al., 2002).
**T serotyping**

The 985 iGAS blood culture isolates from 1995 to 2004 were found to be distributed across a total of 41 different T serotypes. The different serotypes obtained agglutinated either with a single serum or with multiple sera, in which case all the positive reactions were listed and formed the combination serotype name. The dominant serotypes throughout the period were T28, TB3264, T1, T8 and T12, all agglutinating with a single serum (Fig. 2). Each of the five most common serotypes presented in Fig. 2 made up 5% or more of the total amount of isolates in the 10-year study period, and was among the top five most common types at least five times in the 10 years of the study. Overall, the majority of isolates were concentrated within relatively few serotypes; depending on the year, the most common serotypes shown in Fig. 2 accounted for 55–85% of the isolates. The proportion of T non-typable (NT) isolates varied between 2 and 12% annually.

The serotype distribution of Finnish invasive isolates showed a fluctuating pattern throughout the study period. The fluctuation was evident especially in the proportions of the most common serotypes, T28, T1 and TB3264, and showed that a competition existed between serotypes. The most common serotype was T28; the proportion of isolates reacting with anti-T28 typing serum varied annually between 14 and 48% of the total number during the study period, and increased steadily from 1999 to 2003 (with a statistically significant increase from 1999 to 2000, \( P = 0.027 \)). Comparing these results to those of a study of Finnish isolates from 1988 to 1995, we can locate an earlier peak of T28 between 1992 and 1996 (Muotiala et al., 1997). In contrast, the prevalence of serotype T1 has a temporally different cycle, and the proportion of T1 isolates in Finland has been low since its latest peak in 1997, differing considerably from the trend in many other countries, in which T1/M1 isolates have recently been prevalent continuously (Muotiala et al., 1997; Hoe et al., 1999; O'Brien et al., 2002; Li et al., 2003; Ekelund et al., 2005). Serotype TB3264 was most prevalent in 1999 at a season between the peaks of T28 and T1.

The proportion of serotypes grouped as ‘other type’ varied between 11 and 35% annually (see Fig. 2 caption for the different serotypes in this category). The amount of different
serotypes in this category varied annually, from five (in 1995 and 2002) to 19 (in 2004). The large increase in this category from 2003 to 2004 was firstly due to the increase of serotype T3 and its combinations with serotypes B3264, 13 and 9, and secondly a simultaneous decrease in serotype TB3264 (single serum reaction), showing a shift towards combination serotypes. It should be noted here that the T serotyping method is associated with a certain amount of inaccuracy and that some of the reactions with different T antisera may be non-specific, resulting in unusual T-agglutination patterns. When looking at the combinations and interpreting the results, one should be aware that different combinations of common patterns may in fact represent the same type (Johnson et al., 2006). In reality, the category 'other types' of our study may not be as diverse as it seems.

**emm typing of T28 isolates**

The high proportion of isolates reacting with T28 typing sera led us to investigate the clonality of these strains in greater detail. A total of 316 isolates were emm typed; these were isolates reacting with the T28 antiserum alone (305 isolates) and with T28 in combination with other T typing sera (31 isolates). The combination serotypes were T13/28 (15 isolates), T2/28 (eight isolates), T28/B3264 (four isolates), T28/11 (two isolates), T4/28 (one isolate) and T28/B3264/28/8 (one isolate). The isolates were distributed among six distinct emm types: emm28, emm77, emm53 (including subtypes 53.2 and 53.4), emm87, emm2 and emm4 (Fig. 3).

The correlation of these emm types with serotype T28 has been reported elsewhere, although emm53 has historically been associated more with serotype T3/13/B3264 (Strakova et al., 2005; Johnson et al., 2006; ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/emmsequ/).

The proportion of emm28 isolates was highest in 1995–97 (86–44 % of all T28), declined in 1998–2000 (26–30 %) and then showed a considerable increase again in 2001–2004 (80–96 %), the increase from 2000 to 2001 being statistically significant (P<0.0005). During 1999–2000, the most prominent type was emm77 (43–37 % of all T28 isolates). emm53 (including both subtypes), which was fairly uncommon during the first two study years, increased to 34 % of all T28 in 1998, before again becoming rare by 2001 (with a statistically significant decrease from 2000 to 2001, P=0.023). To summarize, the emm type distribution varied in a cyclical pattern, in which, during periods of low emm28 prevalence, emm types 77 and 53 seemed to emerge and partially replace the emm28 type. In contrast, when emm28 became predominant again, it replaced the other emm types almost completely. The proportion of emm87, emm4 and emm2 remained low throughout the study period.

The M-, T- and emm-type distributions among GAS isolates have been found to vary with time, geographic region and disease spectrum (Johnson et al., 1992; Kaplan et al., 2001; O’Brien et al., 2002; Ikebe et al., 2003; Shulman et al., 2004). Our findings are in agreement with those of a Swedish study of invasive and non-invasive isolates, in which an increased spread of T28 with a high proportion of emm28 genotype was observed earlier in 1996–1997 (Eriksson et al., 2003). Looking at the emm28 group alone, a very different temporal distribution was seen in a study of Canadian isolates, in which the number of emm28 isolates first steadily increased and then decreased over a long period from 1995 to 2002, gently peaking in 1997 (Green et al., 2005a). A study of iGAS isolates from the Czech Republic, a peak in the incidence of emm53.2 and emm53.6 strains was observed; however, this occurred in 2001–2004, thus differing temporally from our observations (Strakova et al., 2005).

Within the group of isolates reacting with the single serum T28, emm28 was the dominant type, but all the other aforementioned emm types were found as well. It is evident that a variety of different clones exists within serotype T28 in Finnish isolates. In contrast, the combination serotypes were mostly emm77 or emm2. Looking at our emm28 isolates,

![Fig. 3. emm types of serotype T28 GAS bacteraemia isolates during 1995–2004, Finland. The numbers represent the annual amounts of serotype T28 isolates. The emm typed isolates included strains reacting with the T28 antiserum alone (n=305) and T28 in combination with other T typing sera (n=31). *Includes emm87, emm2 and emm4; †includes emm53.2 and emm53.4.](image-url)
almost all (99%) of them were of serotype T28 and only two isolates were of a combination serotype T28/11. The use of further discriminatory typing methods, such as PFGE and MLST, as well as the determination of virulence factors and antimicrobial susceptibility, is warranted to answer the question of whether the Finnish emm28 isolates in this study are genetically similar or whether there are different clones within this group. In a Swedish study of T28 emm28 isolates, all isolates had the same MLST sequence type and had PFGE patterns that differed by only one to four bands, showing some genetic diversification (Eriksson et al., 2003). On the other hand, M28 strains have been found to be highly diverse in a study of prophage-associated virulence genes (Green et al., 2005a). Furthermore, without extensive information about the emm types within all serotypes, we cannot exclude the possibility that we might have missed some of the emm28 isolates when concentrating on serotype T28 alone. emm type 28 has been observed to correlate with T-agglutination patterns 28, 4/28, NT, 11/28, 12/28, 8/28, 3/13/B3264 and 4 (Johnson et al., 2006). Therefore, especially the serogroup NT, and also 3/13/B3264, might contain some additional emm28 isolates. Should the emm28 type remain prevalent in Finland, it will be essential to obtain more specific information about the clonality of the isolates in order to trace the epidemiology of these strains.

**Conclusion**

The annual incidence of iGAS bacteraemia in Finland has increased during the last decade and follows the general European trend. A fluctuating pattern in the serotype distribution was evident, especially with the most common types, T28, TB3264 and T1, showing a competition between serotypes. emm typing of the serotype T28 isolates revealed that several genetically distinct clones exist in this group and that the prevalence of type emm28 in this group varied in a cyclical pattern over time. This finding highlights the importance of using emm typing alongside T serotyping in the epidemiologic surveillance of iGAS disease. Characterization of the circulating clones and their change over time remains an essential task. To obtain a more comprehensive view of the epidemiology of iGAS disease and the clonality of isolates, additional typing methods could be used to verify and complement these findings.

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**REFERENCES**


