Superantigen gene profile, *emm* type and antibiotic resistance genes among group A streptococcal isolates from Barcelona, Spain

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Group A streptococcus (GAS) has been described as an emerging cause of severe invasive infections. A retrospective hospital-based study was conducted, including GAS isolates causing invasive or non-invasive infections from January 1999 to June 2003 in Barcelona. Demographic and clinical information on the invasive cases was obtained from medical files. GAS isolates collected from 27 patients with invasive infections and 99 patients with non-invasive infections were characterized by *emm* type and subtype, superantigen (SAg) gene profile (*speA*–*C*, *speF*–*J*, *speL*, *speM*, *ssa* and *smeZ*), allelic variants of *speA* and *smeZ* genes, antibiotic susceptibility and genetic resistance determinants. The most prevalent *emm* type was *emm1* (17.5%), followed by *emm3* (8.7%), *emm4* (8.7%), *emm12* (7.1%) and *emm28* (7.1%). The *smeZ* allele and SAg gene profiles were closely associated with the *emm* type. The *speA2*, *speA3* and *speA4* alleles were found in *emm1*, *emm3* and *emm6* isolates, respectively. Overall, 27.8, 25.4 and 11.9% of isolates were resistant to erythromycin, tetracycline or both agents, respectively. Reduced susceptibility to ciprofloxacin and levofloxacin (MIC 2–4 µg ml⁻¹) was found in 3.2% of isolates. *mef(A)*-positive *emm* types 4, 12 and 75, and *erm(B)*-positive *emm* types 11 and 25 were responsible for up to 80% of the erythromycin-resistant isolates. No significant differences in *emm*-type distribution, SAg gene profile or resistance rates were found between invasive and non-invasive isolates. The SAg and antibiotic resistance genes appeared to be associated with the *emm* type and were independent of the disease type.

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INTRODUCTION

Group A streptococcus (GAS) is a human pathogen that is responsible for a wide array of infections, varying in severity from acute pharyngitis and impetigo to severe invasive infections such as necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) (Cunningham, 2000).

The resurgence and persistence of severe forms of GAS diseases reported since the mid 1980s (Hoge *et al.*, 1993; Stevens, 2002) has motivated intensive research on epidemiological, microbiological and clinical aspects of invasive GAS disease. Several factors have been considered to explain

differences in disease frequency and severity, including changes in the virulence of the bacterium (Banks *et al.*, 2002; Musser *et al.*, 1993) and the role of host immunity (Åkesson *et al.*, 2004; Basma *et al.*, 1999; Kotb *et al.*, 2002; Norrby-Teglund *et al.*, 2000).

Among the many factors involved in the virulence of the pathogen, the M protein and a group of exotoxins known as streptococcal superantigens (SAGs) have received considerable attention. Sequence analysis of the *emm* gene (encoding the M protein) has become an important surveillance tool for investigating the dynamics of GAS infection and more than 150 *emm* gene sequence types and several *emm* subtypes have been documented (Facklam *et al.*, 2002; Li *et al.*, 2003).

SAGs are thought to contribute to the pathogenesis of severe GAS infections by virtue of their potent immunostimulatory activity (Norrby-Teglund *et al.*, 2001). The gene distribution

Abbreviations: GAS, group A streptococcus; NF, necrotizing fasciitis; SAg, superantigen; STSS, streptococcal toxic shock syndrome.

The GenBank/EMBL/DDBJ accession numbers for *emm1.25*, *emm12.27*, *emm83.4*, *smeZ-35*, *smeZ-36*, *smeZ-37*, *smeZ-38* and *smeZ-39* are AY686603, AY742805, AY742806, AY965265, AY965878, AY965879, DQ001538 and DQ001539, respectively.

of SAGs has been used as an additional epidemiological tool to explore genomic heterogeneity and the possible correlation between toxin gene content and disease type (Chatellier *et al.*, 2000; Schmitz *et al.*, 2003; Vlamincx *et al.*, 2003).

To improve our understanding of severe GAS disease in Europe, the Strep-EURO project was recently set up (Lamagni *et al.*, 2005; <http://www.strep-euro.lu.se>). To our knowledge, no studies on GAS epidemiology and SAG distribution, including invasive and non-invasive GAS isolates, have been conducted to date in Spain. Surveillance that includes invasive and non-invasive GAS isolates is important to evaluate epidemiological changes in GAS diseases and to distinguish between virulence properties and the prevalence of a particular GAS strain in the general population (Johnson *et al.*, 2002; O'Brien *et al.*, 2002).

In this study, we report clinical and epidemiological data on invasive and non-invasive GAS infections from a tertiary care hospital in Barcelona. *emm* subtype distribution, SAG gene profiles (*speA*–*C*, *speF*–*J*, *speL*, *speM*, *ssa* and *smeZ*) and allelic variants of *speA* and *smeZ* genes were used to evaluate possible differences between GAS isolates causing invasive or non-invasive infections. The prevalence and mechanisms of macrolide, tetracycline and fluoroquinolone resistance were also determined.

METHODS

Bacterial isolates and patients. This retrospective study included GAS clinical isolates collected from January 1999 to June 2003 at Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), a 645-bed tertiary care hospital covering a population of 300 000. Only one isolate per patient was included. Clinical sample procedures and bacterial identification were performed according to standard methods (Ruoff *et al.*, 2003). All isolates were stored at -80°C in trypticase soy broth supplemented with 10% glycerol until testing.

Invasive infection was defined as the recovery of GAS from sites that are usually sterile, including blood, cerebrospinal, peritoneal, pleural and joint fluids, deep tissue abscesses and a superficial site in clinical association with STSS or NF. STSS was defined according to the consensus definition of the Working Group on Severe Streptococcal Infections (1993). Non-invasive isolates were recovered from sites that are usually non-sterile (skin, wound, ear, genital, throat and other miscellaneous sites) and were included for genetic comparison with invasive isolates. Demographic and clinical data on patient characteristics, course of infection and outcome were recorded from the medical history. Medical records were also examined to assess underlying diseases or conditions that may have predisposed patients to invasive disease, such as skin trauma, injection drug use, varicella infection, alcohol abuse or decreased body defences (malignancy, HIV infection, diabetes and immunosuppressive therapy). The overall fatality rate was assessed 30 days after the date of specimen sampling.

Antimicrobial susceptibility testing. The susceptibility to penicillin G, erythromycin, clindamycin, tetracycline and levofloxacin was determined by using the disc-diffusion method, according to Clinical and Laboratory Standards Institute guidelines (National Committee for Clinical Laboratory Standards, 2003) using commercial discs (Bio-Rad). Macrolide resistance phenotypes [constitutive (cMLSb), inducible (iMLSb) and M phenotype] were determined by using the double-disc test, as described by Seppälä *et al.* (1993). MICs of

penicillin G, cefotaxime and levofloxacin were determined for all isolates by using Etest (ABBioDisk), according to the manufacturer's recommendations.

***emm* typing.** The *emm* gene was amplified and sequenced as described previously (Rivera *et al.*, 2005) and by Beall *et al.* (1996). *emm* type and subtype assignments were determined as described on the Centers for Disease Control and Prevention (CDC) website (<http://www.cdc.gov/ncidod/biotech/strep/doc.htm>).

Detection of toxin genes. GAS isolates were tested for the presence of *speA*, *speC*, *speH*, *speI*, *speJ*, *speL*, *speM*, *ssa* and *smeZ* by PCR, using the primers listed in Table 1. With the exception of *speL*, *speM* and *smeZ*, two different primer pairs were used. PCRs were performed using the following conditions: denaturing for 5 min at 94°C , 30 cycles for 30 s at 94°C , annealing for 30 s at the temperature determined for each primer pair (Table 1) and elongation for 1 min at 72°C , followed by a final elongation step at 72°C for 10 min.

Sequences of all *speA* genes were determined and aligned with published sequences in GenBank. Similarly, the sequences of *smeZ* genes of 95 selected isolates representing all *emm* types and subtypes, including those isolates with different toxin profiles, were also determined.

The presence of *speB*, *speF* and *speG* was determined for all isolates by slot-blot analysis (Schleicher & Schuell). DNA samples were obtained as described by Pan & Fisher (1996). DNA probes used in hybridization assays were obtained by PCR with the same conditions as described above using the primers for *speB*, *speF* and *speG* given in Table 1. Labelling of probes and hybridization and detection were performed using ECL Direct Nucleic Acid Labelling (Amersham Biosciences). Hybridization results were confirmed by PCR in 38 randomly selected isolates. Previously published GAS strains, kindly provided by Dr T. Proft, were used as positive controls for the detection of toxin genes, including FP 4223 (positive for *speG*, *speJ*, *speM* and *smeZ*), FP 5417 (positive for *speA*, *speG*, *speJ*, *speL* and *smeZ*), FP 5971 (positive for *speA*, *speC*, *speG*, *speH*, *speJ* and *smeZ*), 1/5045 (positive for *speA*, *speC*, *speG*, *speJ*, *ssa* and *smeZ*) and 85/167 (positive for *speG*, *speH*, *speI*, *speJ* and *smeZ*) (Proft *et al.*, 2003).

Identification of resistance genes. Resistance determinants *erm*(A) subclass *erm*(TR) [hereafter designated *erm*(TR)], *erm*(B) and *mef*(A) were detected in erythromycin-resistant isolates by PCR with the primers listed in Table 1, using the same conditions as described for the toxin genes, except for *mef*(A) (denaturing for 5 min at 94°C , 30 cycles for 1 min at 94°C , annealing for 1 min at 60°C and elongation for 1 min 30 s at 72°C , followed by 10 min at 72°C). In tetracycline-resistant isolates, *tet*(M) and *tet*(O) were also detected by PCR using primers described by Ng *et al.* (2001). Characterization of mutational changes in *parC*, *parE*, *gyrA* and *gyrB* were determined previously in isolates with a levofloxacin MIC of $\geq 2 \mu\text{g ml}^{-1}$ (Rivera *et al.*, 2005).

Statistical analysis. Chi square or Fisher's exact test was used for statistical analysis. A *P* value of <0.05 was considered significant. The SPSS 11.5 statistical package was used for all analyses.

RESULTS AND DISCUSSION

Clinical findings

A total of 126 isolates were recovered, 27 (21.4%) from patients with invasive infections and 99 (78.6%) from non-invasive cases. Skin and soft tissue infections, in the form of cellulitis (five cases), NF (four cases), thrombophlebitis (one case) and bacterial infection secondary to varicella (one

Table 1. Primers used for amplification of *speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speI*, *speJ*, *speL*, *speM*, *ssa* and *smeZ* genes and for sequencing of *speA* and *smeZ* genes

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
<i>speA</i>	ACT(T/C)AAGAACC(A/G)AGAGATG(G/T)*	CTTTAT(T/C)CTTAG(G/A)TATGAAC*	356	50	Modified. Chatellier <i>et al.</i> (2000)
<i>speA</i>	CCAAGCCAACTTCACAGATC	CTTTAT(T/C)CTTAG(G/A)TATGAAC*	523	50	This study
<i>speB</i>	CAACCAGTTGTAAATCTCT	CTAAGGTTTGATGCCTACAA	762	58.3	Modified. Chatellier <i>et al.</i> (2000)
<i>speC</i>	AAGTGACTCTAAGAAAGACA	TTGAGTATCAATGTTAATG	130	50	Chatellier <i>et al.</i> (2000)
<i>speC</i>	TGTCTTATGAGGCCTCTC	ATCTGATCTAGTCCCTTC	386	50	This study
<i>speF</i>	CGAAATTAGAAAAGAGGAC	GGCTGAGCAAAAGTGTGTG	1193	57.3	This study
<i>speG</i>	GATGAAAATTTAAAGATTAA	GGGGGAGAATAGCACTAGT	648	50	Modified. Chatellier <i>et al.</i> (2000)
<i>speH</i>	AATTCTTATAATACAACCAA	TTAGCTGATTGACACATCTA	615	52	Modified. Chatellier <i>et al.</i> (2000)
<i>speH</i>	CACATATTGATAAGAAAATCTACAGC	GAAATTGAGTTGAGTCTATTCTCTCG	666	59	Igwe <i>et al.</i> (2003)
<i>speI</i>	ATGAGTAGTGTGGGAGTTATTAA	TTATTTATTAAATTTAACTAAG	678	50	Proft <i>et al.</i> (2001)
<i>speI</i>	CTTTGGAGTATTCTCCTCCC	CTCTCTCTGTCACCATGTCC	382	59	This study
<i>speJ</i>	GATAGTGAAAATATTTAAAGACG	GCTCCTATCTTATTAGTCC	639	55	Modified. Proft <i>et al.</i> (2001)
<i>speJ</i>	GATAGTGAAAATATTTAAAGACG	TTATTTAGTCCAAAGGTAAATATC	630	55	Modified. McCormick <i>et al.</i> (2001)
<i>speJ</i>	GTTATAATAATCTTTCATGGGTACGG	CTTTCATGTTTATTGCCATTGATCGC	545	59	Igwe <i>et al.</i> (2003)
<i>speL</i>	TTAGGATGGTTTCTGCGGAAGAGAC	TTCCTCTTTCTCGCCTGAGCCGTG	596	60	Igwe <i>et al.</i> (2003)
<i>speM</i>	GCTCTATACACTACTGAGAGTGTC	CATATCAATCGTTTCATTATCTG	612	56	Igwe <i>et al.</i> (2003)
<i>ssa</i>	AGTCAGCCTGACCCTACTCC	TAAGGTGAACCTCTATAGCT	691	57	Modified. Chatellier <i>et al.</i> (2000)
<i>ssa</i>	GTGCACAATTATTATCGATTAGTG	GGTGAACCTCTATAGCTATAGCTGAAG	723	60	Igwe <i>et al.</i> (2003)
<i>smeZ</i>	TAGAAGTAGATAATAATTCC†	TTAGGAGTCAATTTCTATAT†	629	48.3	Modified. Chatellier <i>et al.</i> (2000)
<i>erm</i> (TR)	TTATAACCGGCAAGGAGAAG	GCTTCAGCACCTGTCTTAATTGAT	678	60	Modified. Reinert <i>et al.</i> (2003)
<i>erm</i> (B)	CGAGTGAAAAAGTACTCAACC	GGCGTGTTTCATTGCTTGATG	617	60	Weber <i>et al.</i> (2001)
<i>mef</i> (A)	TATGACAGCCTCAATGCGT	ACCGATTCTATCAGCAAAGA	1435	60	Modified. Reinert <i>et al.</i> (2003)

*Primers used for sequencing the *speA* gene.†Primers used for sequencing the *smeZ* gene.

Table 2. Distribution of *emm* types according to type of infection, toxin gene profile and macrolide and tetracycline resistance genes

Abbreviations: Ery, erythromycin; Tet, tetracycline. +, Present; –, absent.

<i>emm</i> subtype (no. of strains)	No. invasive/ non-invasive cases	Toxin gene profile										No. of strains resistant to:		Resistance gene (no. of strains)
		<i>speA</i> allele	<i>speC</i>	<i>speG</i>	<i>speH</i>	<i>speI</i>	<i>speJ</i>	<i>speL</i>	<i>speM</i>	<i>ssa</i>	<i>smeZ</i> allele (no. positive/no. tested strains)	Ery	Tet	
<i>emm1.0</i> (11)	0/11	<i>speA2</i>	–	+	–	–	+	–	–	–	(6/6)*	1	0	<i>erm</i> (B) (1)
<i>emm1.25</i> (11)	2/9	<i>speA2</i>	–	+	–	–	+	–	–	–	(6/6)*	0	0	–
<i>emm2.0</i> (2)	1/1	–	+	+	–	–	–	+	+	–	(0/2)	0	0	–
<i>emm2.0</i> (1)	0/1	–	+	+	–	–	–	–	+	–	(0/1)	0	0	–
<i>emm2.0</i> (1)	0/1	–	–	+	–	–	–	–	+	–	(0/1)	0	0	–
<i>emm3.1</i> (10)	4/6	<i>speA3</i>	–	+	–	–	–	–	+	+	(6/6)*	0	0	–
<i>emm3.2</i> (1)	0/1	<i>speA3</i>	–	+	–	–	–	–	+	+	(1/1)*	0	0	–
<i>emm4.0</i> (10)	3/7	–	+	–	–	–	–	–	–	+	<i>smeZ</i> -8 (5/5)	9	0	<i>mef</i> (A) (9)
<i>emm4.2</i> (1)	0/1	<i>speA1</i>	–	–	–	–	–	–	+	–	<i>smeZ</i> -39 (1/1)	0	0	–
<i>emm6.0</i> (3)	2/1	<i>speA4</i>	+	+	–	–	–	–	+	–	(3/3)*	0	0	–
<i>emm9.0</i> (4)	2/2	–	–	+	–	–	–	–	–	–	<i>smeZ</i> -36 (4/4)	0	0	–
<i>emm11.0</i> (7)	1/6	–	+	+	+	+	–	–	–	–	<i>smeZ</i> -30/31 (4/4)	7†	7	<i>erm</i> (B) (7)
<i>emm11.0</i> (1)	0/1	–	+	+	–	–	–	–	–	–	<i>smeZ</i> -30/31 (1/1)	1†	1	<i>erm</i> (B) + <i>tet</i> (M) (1)
<i>emm12.0</i> (3)	0/3	–	–	+	+	+	–	–	–	–	<i>smeZ</i> -3 (3/3)	0	0	–
<i>emm12.0</i> (1)	1/0	–	+	+	+	+	–	–	–	–	<i>smeZ</i> -3 (1/1)	0	0	–
<i>emm12.0</i> (1)	0/1	–	–	+	+	+	–	–	–	+	<i>smeZ</i> -3 (1/1)	1	0	<i>mef</i> (A) (1)
<i>emm12.1</i> (1)	0/1	–	–	+	+	+	–	–	–	–	<i>smeZ</i> -3 (1/1)	1	0	<i>mef</i> (A) (1)
<i>emm12.7</i> (1)	0/1	–	–	+	+	+	–	–	–	–	<i>smeZ</i> -3 (1/1)	1	0	<i>mef</i> (A) (1)
<i>emm12.18</i> (1)	0/1	–	–	+	+	+	–	–	–	–	<i>smeZ</i> -3 (1/1)	0	0	–
<i>emm12.27</i> (1)	0/1	–	–	+	+	+	–	–	–	–	<i>smeZ</i> -3 (1/1)	1	0	<i>mef</i> (A) (1)
<i>emm18.9</i> (1)	1/0	<i>speA1</i>	+	+	–	–	+	+	+	–	<i>smeZ</i> -13 (1/1)	0	0	–
<i>emm22.0</i> (1)	0/1	–	+	+	–	–	–	–	–	+	<i>smeZ</i> -22 (1/1)	1†	1	<i>erm</i> (B) + <i>tet</i> (M) (1)
<i>emm22.0</i> (1)	1/0	–	–	+	–	–	–	+	+	+	<i>smeZ</i> -22 (1/1)	0	1	<i>tet</i> (M) (1)
<i>emm25.2</i> (3)	2/1	–	–	+	+	+	–	–	–	+	<i>smeZ</i> -37 (2/2)	3†	3	<i>erm</i> (B) + <i>tet</i> (M) (3)
<i>emm25.2</i> (1)	1/0	–	+	+	+	+	–	–	–	+	<i>smeZ</i> -37 (1/1)	1†	1	<i>erm</i> (B) + <i>tet</i> (M) (1)
<i>emm28.0</i> (6)	0/6	–	+	+	–	–	+	–	+	–	<i>smeZ</i> -16 (2/2)	2	0	<i>erm</i> (B) (2)
<i>emm28.0</i> (1)	0/1	–	+	+	–	–	+	+	+	–	<i>smeZ</i> -16 (1/1)	0	0	–
<i>emm28.0</i> (1)	0/1	<i>speA2</i>	–	+	–	–	+	–	+	–	<i>smeZ</i> -16 (1/1)	0	0	–
<i>emm28.0</i> (1)	1/0	–	+	+	–	–	+	–	–	–	<i>smeZ</i> -16 (1/1)	0	0	–
<i>emm29.2</i> (1)	0/1	–	–	+	–	–	–	+	+	–	<i>smeZ</i> -17 (1/1)	0	0	–
<i>emm43.5</i> (1)	0/1	–	+	+	–	–	+	–	–	–	<i>smeZ</i> -7 (1/1)	0	1	<i>tet</i> (M) (1)
<i>emm44/61.0</i> (1)	0/1	–	–	+	+	–	+	–	+	+	(0/1)	0	1	<i>tet</i> (M) (1)
<i>emm44/61.0</i> (1)	0/1	–	–	+	–	–	+	–	–	+	<i>smeZ</i> -4 (1/1)	0	0	–
<i>emm49.3</i> (1)	0/1	<i>speA1</i>	–	+	+	+	–	–	–	–	<i>smeZ</i> -3 (1/1)	0	1	<i>tet</i> (M) (1)

Table 2. cont.

emm subtype (no. of strains)	No. invasive/ non-invasive cases	Toxin gene profile										No. of strains resistant to:		Resistance gene (no. of strains)
		speA allele	speC	speG	speH	speI	speJ	speL	speM	ssa	smeZ allele (no. positive/no. tested strains)	Ery	Tet	
emm50.0 (2)	1/1	—	+	+	—	—	—	—	—	—	smeZ-33 (1/1)	0	0	—
emm50.0 (1)	0/1	—	—	+	—	—	—	—	+	+	smeZ-35 (1/1)	0	1	tet(M) (1)
emm58.0 (1)	0/1	—	—	+	—	—	—	—	—	+	smeZ-28 (1/1)	1†	1	erm(TR) + tet(M) (1)
emm58.0 (1)	0/1	—	+	+	+	—	—	—	—	—	smeZ-28 (1/1)	0	0	—
emm59.0 (2)	0/2	—	—	+	—	—	+	—	+	—	smeZ-12 (2/2)	0	2	tet(M) (2)
emm63.0 (1)	0/1	—	—	—	—	—	—	—	—	—	(0/1)	0	1	tet(M) (1)
emm64.0 (2)	0/2	—	—	+	—	—	—	—	+	—	smeZ-4 (1/2)	0	0	—
emm70.0 (1)	0/1	—	—	+	—	—	—	+	+	—	smeZ-17 (1/1)	0	1	tet(M) (1)
emm75.0 (2)	0/2	—	+	+	+	+	+	+	+	—	smeZ-29 (2/2)	2	0	mef(A) (2)
emm75.0 (1)	0/1	—	+	+	+	+	—	+	+	—	smeZ-29 (1/1)	1	0	mef(A) (1)
emm77.0 (5)	1/4	—	+	—	—	—	—	—	—	—	smeZ-37 (5/5)	1†	5	erm(TR) + tet(O) (1) tet(M) + tet(O) (1) tet(O) (3)
emm77.0 (1)	1/0	—	+	+	—	—	—	—	—	—	smeZ-37 (1/1)	0	1	tet(O) (1)
emm77.0 (1)	0/1	—	+	—	+	—	—	—	—	—	smeZ-37 (1/1)	0	0	—
emm77.0 (1)	1/0	—	+	—	+	—	—	—	—	+	smeZ-37 (1/1)	0	1	tet(O) (1)
emm81.0 (1)	0/1	—	—	+	+	—	—	—	—	—	(0/1)	1	0	mef(A) (1)
emm83.4 (1)	1/0	—	+	+	—	—	—	+	+	—	smeZ-15 (1/1)	0	1	tet(M) (1)
emm87.0 (3)	0/3	—	+	+	—	—	+	—	—	+	smeZ-5 (2/2)	0	0	—
emm89.0 (5)	0/5	—	—	+	—	—	—	—	—	—	smeZ-13 (4/4)	0	0	—
st11014 (1)	0/1	—	+	—	+	—	—	—	—	—	smeZ-38 (1/1)	0	1	tet(M) (1)
Total no.‡	27/99	40	56	106	30	24	42	11	41	36	88	35	32	
Percentage	21·4/78·6	31·7	44·4	84·1	23·8	19	33·3	8·7	32·5	28·6	91·7	27·8	25·4	

*Nucleotide sequences of the *smeZ* alleles of the *emm1*, *emm3* and *emm6* strains tested were identical to those of the *smeZ* allele of strains M1 GAS (GenBank accession no. AE006622), MGAS315 (AE014169) and MGAS10394 (NC_006086), respectively. A specific designation has not been attributed to these alleles.

†Also resistant to tetracycline.

‡Total number and frequency of each toxin gene in the entire collection of strains, except for *smeZ*, which was tested in 96 of 126 strains.

case), were the most frequent clinical manifestations of invasive cases (11 of 27; 40.7%). Other diagnoses of invasive infections included arthritis (five cases), bacteraemia without specific focus (four cases), pneumonia (three cases), and one case each of meningitis, peritonitis, endocarditis and psoas abscess. STSS was recorded in four of the invasive cases (two cases of NF, one case of endocarditis and one case of bacteraemia without specific focus). The clinical presentations or sources of isolation of the 99 non-invasive isolates were skin-wound infections and impetigo ($n=37$), genital infections ($n=20$), otitis ($n=19$), abscesses ($n=11$; 10 cutaneous and one tonsillar), pharyngitis ($n=10$) and other sources including urine and eyes ($n=2$).

The median age of patients with invasive infections was 55 years (mean 51.2 years; range 10 months to 89 years), whereas in the non-invasive group the median age was 8 years (mean 22.9 years; range 1 month to 91 years). The male to female ratio was 1.5:1 and 1:1 for the invasive and non-invasive groups, respectively. At least one comorbid disease or local predisposing factor was found in 20 (80%) out of 25 patients with available data in the invasive group. In one of the paediatric patients, invasive infection was associated with varicella, whereas, among adult patients, underlying conditions included skin trauma (eight cases), intravenous drug use and HIV infection (four cases, two with STSS and NF), cancer (four cases), peripheral vascular disease (three cases), immunosuppressive therapy (two cases), alcohol abuse (two cases) and diabetes mellitus (one case). Mortality occurred in three of 27 (11%) patients with invasive infections, two of whom had STSS. Therefore, in agreement with previously published reports (Kao *et al.*, 2005; O'Brien *et al.*, 2002), most patients with invasive GAS infection were adults and a large proportion had underlying conditions, with HIV infection and injection drug use among the most frequent. Invasive GAS infection associated with injection drug use appears to be increasing in some countries (Efstratiou *et al.*, 2003) and epidemics among this group have been documented (Lécho *et al.*, 2001). It should be noted that, during the period studied, a local outbreak of invasive disease was detected among injecting drug users (Sierra *et al.*, 2006). In this work, all invasive *emm25.2* isolates were isolated from injecting drug users and were part of the local outbreak. Interestingly, an outbreak of invasive infection among drug users in Switzerland was reportedly caused by a specific *emm25* clone (Bohlen *et al.*, 2000).

Distribution of *emm* types

In total, 29 *emm* types were identified among the 126 isolates (Table 2), of which *emm1* (22 isolates, 17.5%) was the most prevalent, followed by *emm3* (11 isolates, 8.7%), *emm4* (11 isolates, 8.7%), *emm12* (nine isolates, 7.1%), *emm28* (nine isolates, 7.1%), *emm11* (eight isolates, 6.3%) and *emm77* (eight isolates, 6.3%). These seven *emm* types accounted for 61.9% of isolates and have been reported to be among the most prevalent in USA, Canada and Europe (O'Brien *et al.*, 2002; Schmitz *et al.*, 2003; Tyrrell *et al.*, 2002; Vlamincx *et al.*, 2003).

Most isolates presented *emm* sequences that were identical to those of the corresponding CDC *emm* reference strains. Seven types (*emm3*, *emm18*, *emm25*, *emm29*, *emm43*, *emm49* and *emm83*) were exclusively represented by allelic variants of the reference strains. Different subtypes within a type were only found in types *emm1*, *emm3*, *emm4* (two subtypes each) and *emm12* (five subtypes). Three subtypes designated *emm1.25*, *emm12.27* and *emm83.4* were described for the first time in this study.

A total of 15 different *emm* types were identified in the 27 invasive isolates. All but two represented by single isolates were also present among non-invasive isolates. The most prevalent *emm* types were *emm3* (14.8%), *emm4* (11.1%), *emm25* (11.1%), *emm77* (11.1%), *emm1* (7.4%), *emm6* (7.4%) and *emm9* (7.4%). A diversity of *emm* types was detected in isolates from the six patients with the most severe forms of infection (NF and SSTs), including two *emm25* and one each of *emm1*, *emm4*, *emm11* and *emm22*. In the 99 non-invasive isolates, 27 different *emm* types were found, the most prevalent being *emm1* (20.2%), *emm4* (8.1%), *emm12* (8.1%), *emm28* (8.1%), *emm3* (7.1%) and *emm11* (7.1%).

No significant differences in *emm* type distribution were found between invasive and non-invasive isolates, as has been described recently (Descheemaeker *et al.*, 2000; Haukness *et al.*, 2002; Ho *et al.*, 2003; Johnson *et al.*, 2002; Kao *et al.*, 2005). However, earlier reports identified a shift in dominant serotypes coincident with an increase in severe GAS infections, with M1 and M3 being those most frequently isolated from invasive cases (Johnson *et al.*, 1992; Schwartz *et al.*, 1990). The emergence of clones with increased virulence was proposed to explain the observed changes in GAS epidemiology (Musser *et al.*, 1993, 1995).

Over the study period, there were no annual variations in the prevalence of the most common *emm* types. Certain subtypes such as *emm11.0*, *emm25.2* and the newly identified *emm1.25* were only found in years 2002 and 2003.

Distribution of SAGs and toxin gene profiles

Among the toxin genes that are thought to be chromosomally encoded, *speB* and *speF* were found in all isolates. As shown in Table 2, *speG* was found in 106 (84.1%) isolates and *speJ* was only found in 42 (33.3%) isolates. *smeZ* was identified in 88 (91.7%) of 96 isolates tested. DNA sequence analysis revealed the presence of 23 different *smeZ* alleles. Five *smeZ* alleles designated *smeZ-35*–*smeZ-39* were identified for the first time in this study. As described by Proft *et al.* (2000), there was a strong association between the *smeZ* allele and the *emm* type. Five *smeZ* alleles (*smeZ-3*, *smeZ-4*, *smeZ-13*, *smeZ-17* and *smeZ-37*) were each found in isolates of two different *emm* types. Isolates within an *emm* type carried the same *smeZ* allele except for the *emm50* and *emm4* isolates (Table 2). The genes *speA*, *speC*, *speH*, *speI*, *speL*, *speM* and *ssa*, which have been found to be associated with prophage elements, were present at variable frequencies

(Table 2). Significant differences were only found for *ssa*, which was more frequent in isolates from invasive cases ($P < 0.05$, Odds' ratio 2.5; 95 % confidence interval, 1.27–4.91). This correlation probably reflects the association of the *ssa* gene with the most prevalent *emm* types found in invasive infections (*emm3*, *emm25* and *emm4*). Sequence analysis of the *speA* gene revealed four different alleles (Nelson *et al.*, 1991), with a limited distribution of *speA2*, *speA3* and *speA4* in *emm1*, *emm3* and *emm6* isolates, respectively. The *speA1* allele was identified in single isolates of three distinct *emm* types (*emm18*, *emm4* and *emm49*).

The analysis revealed that isolates of the same *emm* type shared a common toxin gene profile (*emm* types 1, 3, 6, 9, 59, 64, 87 and 89) or a predominant numerical profile (Table 2). Differences in toxin gene profiles between isolates of the same *emm* type involved the presence or absence of only one or two toxin genes compared with the dominant profile, except for the *emm4.2* isolate, which differed in four toxin genes from *emm4.0* isolates.

Although the number of isolates studied was limited, no significant difference in toxin gene profile was found between invasive and non-invasive isolates. Two recent European studies involving a larger number of isolates found that, despite the presence of several different toxin gene profiles within an *emm* type, each *emm* type was characterized by a predominance of one or two toxin gene profiles, reflecting the spread of few invasive clones throughout the world (Schmitz *et al.*, 2003; Vlamincx *et al.*, 2003).

Antimicrobial susceptibility

All isolates were susceptible to penicillin G and cefotaxime. Erythromycin resistance was found in 35 isolates (27.8 %), tetracycline resistance in 32 isolates (25.4 %) and resistance to both agents in 15 isolates (11.9 %). Four isolates (3.2 %) showed reduced susceptibility to levofloxacin (MIC 2–3 $\mu\text{g ml}^{-1}$).

Seventeen (48.6 %) of the erythromycin-resistant isolates expressed the M phenotype and all of them harboured *meffA*. The cMLSb phenotype was observed in 15 (42.8 %) isolates, in association with *erm(B)* in all but one, which carried *erm(TR)*. Three (8.6 %) isolates presented iMLSb; two of these carried *erm(B)* and the third carried *erm(TR)*. The most prevalent *emm* types of erythromycin-resistant isolates were *emm4*, *emm11*, *emm12* (four subtypes), *emm25* and *emm75*. Together, these five *emm* types represented 80 % of the erythromycin-resistant isolates, but only 7.7 % of the susceptible isolates. All the *emm4*, *emm12* and *emm75* erythromycin-resistant isolates harboured the *meffA* gene, whereas all *emm11* and *emm25* isolates carried the *erm(B)* gene. These data suggested that the high rate of erythromycin resistance was caused by the spread of a limited number of clones. An association between certain *emm* types and erythromycin resistance or macrolide resistance mechanisms has been documented (Albertí *et al.*, 2003;

Zampaloni *et al.*, 2003) and may possibly reflect the spread of resistant clones. In Spain, a strong association has been reported between *emm* types 4 and 75 and erythromycin resistance (Albertí *et al.*, 2003; Pérez-Trallero *et al.*, 1999).

A total of 32 tetracycline-resistant isolates were detected, belonging to 14 different *emm* types. The resistance determinant *tet(M)* was present in 18 isolates (56.2 %), *tet(O)* in six isolates (18.7 %), and one isolate carried both genes. None of the remaining seven tetracycline-resistant isolates carried either *tet(M)* or *tet(O)*. *tet(O)* was found exclusively in *emm77.0* isolates.

Among the 15 isolates that were resistant to both erythromycin and tetracycline, *erm(B)* and *tet(M)* were found in six isolates, *erm(TR)* and *tet(O)* in one isolate and *erm(TR)* and *tet(M)* in one isolate. Seven isolates, all *emm11*, carried *erm(B)*, but none of the tetracycline resistance genes studied. None of the strains presenting the M phenotype were resistant to tetracycline.

The frequency of erythromycin-resistant isolates showed a rising trend over the study period (16.6 % in 1999, 19 % in 2000, 21 % in 2001, 34.6 % in 2002 and 38.8 % from January to June 2003). A high frequency of erythromycin resistance has been found in Spain (29.7 %) (Alós *et al.*, 2003) and other European countries, including Italy (35.8 %; Dicuonzo *et al.*, 2002) and France (22.4 %; Bingen *et al.*, 2004), in contrast with the low rates observed in other countries such as the USA (6.8 %; Richter *et al.*, 2005). The frequency of tetracycline resistance fluctuated year-to-year (33.3 % in 1999, 19 % in 2000, 15.8 % in 2001, 34.6 % in 2002 and 24.2 % from January to June 2003). Multiclonal dissemination and high rates of tetracycline resistance have been reported from other countries (de Melo *et al.*, 2003; Jasir *et al.*, 2000).

The proportions of resistant isolates were similar in the invasive and non-invasive groups for both erythromycin (22.2 % invasive versus 29.3 % non-invasive) and tetracycline (33.3 % invasive versus 23.2 % non-invasive).

A number of reports during the last few years have described an increased prevalence of isolates with reduced susceptibility to fluoroquinolones (ciprofloxacin MIC $\geq 2 \mu\text{g ml}^{-1}$) (Albertí *et al.*, 2005; Malhotra-Kumar *et al.*, 2005; Powis *et al.*, 2005). In the present study, four isolates of three different *emm* types (*emm3.1*, *emm6.0* and *emm12.27*) showed reduced susceptibility to levofloxacin (MIC 2–3 $\mu\text{g ml}^{-1}$) and carried mutational changes in *parC*. A detailed characterization of these isolates has been published previously (Rivera *et al.*, 2005).

In conclusion, the overall *emm*-type distribution found in this study was comparable with previous reports from North America and Europe. We found no differences in *emm* type distribution among isolates causing invasive or non-invasive infection. In addition, isolates of the same *emm* type presented restricted genetic variation with respect to SAg

profiles. The erythromycin resistance rate presented a rising trend over the period studied and involved the spread of a limited number of clones. The *Sag* and antibiotic resistance genes thus appeared to be associated with the *emm* type and were independent of the disease type.

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