Genetic diversity and virulence properties of *Streptococcus dysgalactiae* subsp. *equisimilis* from different sources

Giovanni Gherardi,1 Monica Imperi,2 Claudio Palmieri,3 Gloria Magi,3 Bruna Facinelli,3 Lucilla Baldassarri,2 Marco Pataracchia2 and Roberta Creti2

1Dipartimento di Centro Integrato di Ricerca (CIR), Università Campus Bio-Medico, Rome, Italy
2Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Rome, Italy
3Dipartimento di Scienze Biomediche e Sanità Pubblica, Sezione Microbiologia, Università Politecnica delle Marche, Ancona, Italy

A recent increase in virulence of pathogenic *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) has been widely proposed. Such an increase may be partly explained by the acquisition of new virulence traits by horizontal gene transfer from related streptococci such as *Streptococcus pyogenes* (GAS) and *Streptococcus agalactiae* (GBS). A collection of 54 SDSE strains isolated in Italy in the years 2000–2010 from different sources (paediatric throat carriage, invasive and non-invasive diseases) was characterized by *emm* typing and pulsed-field gel electrophoresis (PFGE) analysis. The virulence repertoire was evaluated by PCR for the presence of GAS superantigen (*spe*) genes, the streptolysin S (*sagA*) gene, the group G fibronectin-binding protein (*gfbA*) gene and GAS–GBS alpha-like protein family (*alp*) genes; moreover, the ability to invade human epithelial cells was investigated. Resistance to tetracycline, erythromycin and clindamycin was assessed. The combined use of *emm* typing and PFGE proved to be a reliable strategy for the epidemiological analysis of SDSE isolates. The most frequent *emm* types were the same as those more frequently reported in other studies, thus indicating the diffusion of a limited number of a few successful *emm* types fit to disseminate in humans. The *speG* gene was detected in SDSE strains of different genetic backgrounds. Erythromycin resistance determined by the *erm*(T) gene, and the unusual, foggy MLSB phenotype, observed in one and seven strains, respectively, have never previously, to our knowledge, been reported in SDSE. Moreover, a new member of the *alp* family was identified. The identification of new antibiotic and virulence determinants, despite the small size of the sample analysed, shows the importance of constant attention to monitoring the extent of lateral gene transfer in this emerging pathogen.

INTRODUCTION

Genetic studies on beta-haemolytic, large-colony-forming, human streptococci belonging to Lancefield groups G and C have demonstrated they are members of a single streptococcal subspecies, *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) (Vandamme et al., 1996). Recently, SDSE has increasingly been reported as an important emerging human pathogen, being responsible for infections similar to those caused by *Streptococcus pyogenes* (group A streptococcus, GAS), such as pharyngitis, skin and soft tissue infection, toxic shock syndrome, bacteraemia, reactive arthritis, post-infectious glomerulonephritis and acute rheumatic fever (Brandt & Spellerberg, 2009).

Recent whole-genome analyses of sequenced SDSE isolates demonstrated a high rate of overlap (61–63 %) between SDSE and GAS genomes. SDSE shares several virulence factors with GAS, including M protein (encoded by *emm*-like genes), capsule, surface proteins, streptokinase, streptolysin O and S, pyrogenic exotoxin G, and fibronectin-binding proteins, probably as a result of interspecies genetic transfer (Shimomura et al., 2011; Suzuki et al., 2011; Choi et al., 2012).

Abbreviations: GAS, group A streptococcus; GBS, group B streptococcus; HI, highly invasive; NI, non-invasive; PFGE, pulsed-field gel electrophoresis; SDSE, *Streptococcus dysgalactiae* subsp. *equisimilis*; WI, weakly invasive.

A supplementary table is available with the online version of this paper.
A valuable epidemiological tool for SDSE is emm typing, often used in association with macrorestriction profiling by means of pulsed-field gel electrophoresis (PFGE) (Pinho et al., 2006; McDonald et al., 2007; Ahmad et al., 2009; Broyles et al., 2009; Rantala et al., 2010). Like GAS, SDSEs are able to invade in vitro human respiratory epithelial cells thanks to fibronectin-binding proteins, such as GfaA/SfbI, acting as invasins (Haidan et al., 2000; Palmieri et al., 2007). High rates of macrolide resistance encoded by erm (erythromycin ribosome methylase) and mef (macrolide efflux) genes have been reported since the 1990s among major streptococcal pathogens and, more recently, in SDSE (Brandt & Spellerberg, 2009; Broyles et al., 2009).

In the present work we investigated the genetic relationships, the virulence profile and cell invasiveness, as well as the susceptibility to macrolide and tetracycline antibiotics of SDSE strains isolated throughout Italy from invasive and non-invasive diseases and from carriers.

METHODS

Bacterial strains. A total of 54 SDSE isolates was received by hospitals during national enhanced passive surveillance programmes from 2000 to 2010. Isolates were recovered from 17 cases of invasive disease (bacteraemia, n=15; septic arthritis, n=1; intravascular catheter-related infection, n=1), 26 cases of non-invasive disease (wounds, n=19; urinary tract infection, n=1; abscess, n=1; respiratory tract infection, n=3; ear infection, n=1; joint infection, n=1) and from the pharynges of asymptomatic children (11 isolates). Bacterial identification was accomplished, upon receipt, by determining the Lancefield group using the commercial Dryspot streptococcal grouping kit (Oxoid), and identification to the species mining the Lancefield group using the commercial Dryspot Bacterial identification was accomplished, upon receipt, by deter-

DNA isolation and PCR screening for virulence profile. Total bacterial DNA was prepared by a Chelex-based procedure using an InstaGene Matrix (Bio-Rad). Briefly, a large loop from a single colony grown overnight was dissolved in a tube containing 1 ml sterile distilled water and then treated according to the manufacturer’s instructions. The isolates were investigated by PCR for the presence of the superantigen genes speA, speB, speC, speG, speH, speL, speM, ssa and smeZ (Igwe et al., 2003; Hashikawa et al., 2004), for the streptolysin S gene (sagA) (Igwe et al., 2003), for alpha-like protein (alp) genes (Creti et al., 2004) and for the fibronectin-binding protein gfbA gene (Rohde et al., 2011).

Erythromycin, clindamycin and tetracycline resistance determinants. Resistance to erythromycin and clindamycin was assessed phenotypically by both E-test (BIODISK) for MIC value determination and the Kirby–Bauer double disc diffusion method to assign the constitutive macrolide–lincosamide–streptogramin B (cMLSb), inducible MLSb (iMLSb) and macrolide (M) resistance phenotypes (Varaldo et al., 2009). The presence of the macrolide resistance genes erm(A) [subclass erm(TRY)], erm(B) and mef was investigated in a multiplex PCR, as already described (Creti et al., 2007a). The mef amplicon was sequenced for the identification of the mef class. PCR conditions and primer sequences used for amplification of the emr(T) gene were as previously described (DiPersio et al., 2011) and the amplicon was then sequenced to confirm its identity. Tetracycline resistance was determined both phenotypically by E-test and genotypically by studying the occurrence of the resistance genes tet(M) and tet(O) (Malhotra-Kumar et al., 2005). In the case of a phenotypic tetracycline-resistant strain [tet(M) and tet(O) genes negative] a PCR screening for tet(K), tet(L) (Malhotra-Kumar et al., 2005), tet(S) (Ng et al., 2001) and tet(W), including hybrid tet genes, was also performed (Stanton & Humphrey, 2003).

emm typing. Isolates were emm typed according to protocols and recommendations by CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). According to the CDC guidelines, both the emm type and subtypes were assigned by the comparison of the query sequence with the reference database. Assignment of emm type is based upon the 90 bases encoding the N-terminal 30 residues of the processed M protein; emm subtypes are assigned according to the exact 150-base sequence encoding the N-terminal 50 residues of the mature M protein.

PFGE analysis. Total DNA was extracted and digested with 40 U of Smal. DNA bands were resolved and interpreted according to previously reported criteria (van Belkum et al., 2007): isolates with identical profiles were assigned to the same PFGE type and subtype; isolates with similar profiles (differing by one to four bands) were assigned to different subtypes within the same PFGE type; isolates with more than four bands different were assigned to unrelated PFGE types.

Cell-invasion assay. The ability to enter human respiratory cells was examined by means of the human alveolar carcinoma cell line A549 (ATCC CCL-185). Cell-invasion assays were done as previously described for GAS (Facinelli et al., 2001). Each SDSE strain was tested in three separate assays on different days; each assay represented the mean of triplicate wells. Cell-invasion efficiency was established on the basis of the percentage of survivors (relative to initial inoculum) recovered after the incubation of infected cells with penicillin and gentamicin which are unable to reach intracellular bacteria. Depending on cell-invasion efficiency (>10%, 1–10% or <1%), strains were defined as highly invasive (HI), weakly invasive (WI) or non-invasive (NI), as previously described for GAS (Facinelli et al., 2001).

Statistical analysis. Fisher’s exact test was used to evaluate the differences in distribution of isolates. Two-sided P-values <0.01 were considered statistically significant (SPSS 17 for Windows).

RESULTS

Clonal relatedness among isolates defined by emm typing and PFGE

By latex agglutination of Lancefield group polysaccharide, the 54 SDSE strains used in this study were classified as group G (39 isolates) and group C (15 isolates). The emm gene was successfully amplified in all isolates. A total of 18 different emm types was observed, with 25 different emm subtypes (Table 1 and Table S1, available in JMM Online). Four new emm subtypes were detected (stG10.3, stG10.4, stG480.7, stG6792.4). Overall, ten emm types, accounting for 83.3% of the isolates, were shared by more than one isolate and eight unique emm types were found; emm type stG6, consisting of two subtypes and nine isolates, was the most prevalent, followed by stC6792, represented by six isolates, stG480 and stG62647 (five isolates each), stG6792, stC839, stG485 and stG643 (four isolates each) (Table 1 and Table S1).

Overall, seven multiple-strain emm types were found exclusively among either group G (emm types stG6, stG6792,
Table 1. Genotypic and phenotypic characteristics of 54 *Streptococcus dysgalactiae* subsp. *equisimilis* strains isolated from human invasive disease, non-invasive disease and carriers, Italy

<table>
<thead>
<tr>
<th><em>emm</em> type (n)</th>
<th>PFGE type (n)</th>
<th>Lancefield group (n)</th>
<th>Strain type (n)*</th>
<th>Virulence genes (n)</th>
<th>Macrolide resistance genotype/phenotype (n)</th>
<th>Tetracycline resistance gene (n)</th>
<th>Cell invasiveness†</th>
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<tr>
<td><em>stG6</em> (9)</td>
<td>7 (5), 13 (2), ND (2)</td>
<td>G (9)</td>
<td>nInv (5), C (4)</td>
<td>speG (2)</td>
<td>erm(A)/cMLSB (1), erm(B)/cMLSB (1), mef(A)/M (2)</td>
<td>–</td>
<td>HI (5), WI (1), NI (3)</td>
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<tr>
<td><em>stG6979</em> (6)</td>
<td>16 (6)</td>
<td>G (6)</td>
<td>Inv (1), nInv (5)</td>
<td>–</td>
<td>erm(A)/iMLSB (6)</td>
<td>tet(M) (4)</td>
<td>HI (1), WI (1), NI (3)</td>
</tr>
<tr>
<td><em>stG480</em> (5)</td>
<td>4 (5)</td>
<td>G (5) C (1)</td>
<td>Inv (2), nInv (2)</td>
<td>speG (3), R28 (1)</td>
<td>–</td>
<td>–</td>
<td>NI (5)</td>
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<tr>
<td><em>stG62647</em> (5)</td>
<td>2 (5)</td>
<td>C (3), G (2)</td>
<td>Inv (2), nInv (3)</td>
<td>speG (4)</td>
<td>–</td>
<td>–</td>
<td>HI (3), NI (1)</td>
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<tr>
<td><em>stG6792</em> (4)</td>
<td>1 (4)</td>
<td>G (4)</td>
<td>Inv (3), nInv (1)</td>
<td>speG (1)</td>
<td>–</td>
<td>tet(M)</td>
<td>WI (2), NI (2)</td>
</tr>
<tr>
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<td>2 (2), 3 (2)</td>
<td>G (4)</td>
<td>Inv (2), nInv (1), C (1)</td>
<td>speG (2), Dysalp (1)</td>
<td>–</td>
<td>–</td>
<td>WI (2), NI (2)</td>
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<tr>
<td><em>stG839</em> (4)</td>
<td>5 (3), 9 (1)</td>
<td>C (3), G (1)</td>
<td>nInv (3), C (1)</td>
<td>speG (1)</td>
<td>erm(A)/iMLSB (2)</td>
<td>tet(M) (1)</td>
<td>WI (2), NI (2)</td>
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<tr>
<td><em>stG643</em> (4)</td>
<td>2 (1), 8 (1), 20 (1), ND (1)</td>
<td>G (4)</td>
<td>nInv (3), C (1)</td>
<td>speG (3), R28 (1)</td>
<td>mef(E)/M (1)</td>
<td>tet(M) (2), (1)</td>
<td>WI (1), NI (3)</td>
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<tr>
<td><em>stC36</em> (3)</td>
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<td>C (2), G (1)</td>
<td>Inv (1), nInv (1), C (1)</td>
<td>–</td>
<td>mef(E)/M (1)</td>
<td>–</td>
<td>HI (2), NI (1)</td>
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<td>G (2)</td>
<td>Inv (2)</td>
<td>speG (1), R28/speG (1)</td>
<td>erm(A)/iMLSB (1)</td>
<td>tet(M) (2)</td>
<td>NI (2)</td>
</tr>
<tr>
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<td>G</td>
<td>Inv</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NI</td>
</tr>
<tr>
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<td>6</td>
<td>G</td>
<td>nInv</td>
<td>speG</td>
<td>erm(T)/iMLSB</td>
<td>tet(M)</td>
<td>NI</td>
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<tr>
<td><em>stG245</em> (1)</td>
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<td>G</td>
<td>Inv</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>WI</td>
</tr>
<tr>
<td><em>stC5345</em> (1)</td>
<td>14</td>
<td>G</td>
<td>C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NI</td>
</tr>
<tr>
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<td>15</td>
<td>G</td>
<td>C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NI</td>
</tr>
<tr>
<td><em>stG2078</em> (1)</td>
<td>17</td>
<td>G</td>
<td>Inv</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>WI</td>
</tr>
<tr>
<td><em>stG653</em> (1)</td>
<td>18</td>
<td>C</td>
<td>nInv</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NI</td>
</tr>
<tr>
<td><em>stG11</em> (1)</td>
<td>19</td>
<td>G</td>
<td>Inv</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NI</td>
</tr>
</tbody>
</table>

*Strain types were defined based upon either clinical manifestation or carriage status and are represented by invasive (Inv), non-invasive (nInv) and colonizing (C) isolates.
†Isolates were classified based upon cell invasiveness as high invasive (HI), weak invasive (WI) and non-invasive (NI).
‡This strain was tetracycline-resistant but PCR-negative for all *tet* genes studied.
stG10, stG480, stG643 and stG485) or group C (emm type stC6979) isolates (Table 1 and Table S1). The other three emm types (stG62647, stC36 and stC839) represented by two or more isolates included both G and C group isolates (Table 1 and Table S1).

PFGE analysis resolved all but three SDSE strains. A total of 20 different PFGE types was identified, ten of which were shared by more than one isolate (Tables 1 and S1). Overall, good concordance between emm type and PFGE could be observed. Seven out of ten multiple-strain PFGE groups (PFGE types 4, 5, 7, 10, 12, 13 and 16) comprised unique emm types (Fig. 1a) and, in some cases, emm and PFGE subtypes could further resolve small subclones, as was the case of stG6 isolates with three different emm subtypes associated with specific PFGE subtypes (Fig. 1b).

Nevertheless, three out of ten multiple-strain PFGE groups (namely PFGE types 1–3) included more than one emm type, with PFGE type 2 being the most heterogeneous, comprising three different emm type strains (stG62647, stG485 and stG643) (Fig. 1a). In addition, in five cases the same emm types could be found in more than one PFGE group: stG6 and stG643, dispersed among three distinct PFGE types each, and stG485, stC839 and stC36, represented in two different PFGE groups (Fig. 1a).

Detection of virulence genes

A total of 12 virulence genes was assessed by PCR. All strains were negative for speA, speC, speH, speJ, speL, speM, ssa and smeZ genes but all possessed the sagA gene. The spe gene was detected in 19 strains (Table 1 and Table S1). alp genes were identified in four strains, three possessing the GAS–GBS R28 gene and one carrying a new member of the family, dysalp, first detected in SDSE and recently described (Creti et al., 2007b). As shown in Table 1 and Table S1, four different virulence gene profiles were identified.
Detection of erythromycin, clindamycin and tetracycline resistance determinants

Overall, 17 out of 54 isolates (31.5%) were erythromycin-resistant, 13 of which (76.5%) also displayed resistance to clindamycin (MLS<sub>B</sub> phenotype). In particular, seven strains showed the ambiguous foggy MLS<sub>B</sub> phenotype (FMMLS<sub>B</sub>), characterized by a blunt inhibitory zone around the clindamycin disc proximal to the erythromycin disc, with scarce bacterial growth; three strains displayed the constitutive (cMLS<sub>B</sub>) phenotype and three the inducible (iMLS<sub>B</sub>) resistance phenotype. All strains possessed <i>erm</i> genes, with <i>erm</i>(A), <i>erm</i>(B) and <i>erm</i>(T) genes recovered in 11, 1 and 1 isolates, respectively. All seven isolates with the FMMLS<sub>B</sub> phenotype carried the <i>erm</i>(A) resistance gene; of these, six strains were part of a clonal cluster (<i>emm</i> type stG6979 and PFGE 16) and one was <i>emm</i> type stG10 and belonged to PFGE type 10 (Table 1 and Table S1). Among the four erythromycin-resistant, clindamycin-susceptible isolates (M phenotype) (23.5%), two strains possessed <i>mef</i>(A) and two <i>mef</i>(E). Resistance to tetracycline was found in 15 isolates (27.8%); 14 of these carried the tet(M) gene, one the tet(O) gene, and one was negative for all tet genes tested [(tet(M), tet(O), tet(K), tet(L), tet(S), tet(W)]). Seven isolates were resistant to both erythromycin and tetracycline and carried <i>erm</i>(A) and tet(M) genes (five isolates), <i>erm</i>(T) and tet(M) genes (one isolate), and <i>mef</i>(E) and tet(M) genes (one isolate).

Cell invasiveness and characteristics of HI, WI and NI SDSE strains

In internalization assays, 11/54 strains were HI and 9/54 were WI, the remaining 34 being NI (Table 1 and Table S1). A significant association (P<0.001) between high cell-invasion efficiency and the presence of <i>gfbA</i> was detected. HI strains were all <i>gfbA</i>-positive, belonged to four different <i>emm</i> types (stG36, stG480, stG6 and stG6792, Fig. 2a) and were from both invasive (<i>n</i>=4) and non-invasive (<i>n</i>=4) infections and from carriers (<i>n</i>=3). All but two HI strains were erythromycin-susceptible. Ten (23.3%) WI/NI strains harboured <i>gfbA</i> and belonged to seven <i>emm</i> types from non-invasive (<i>n</i>=6) or invasive (<i>n</i>=3) infections and from carriers (<i>n</i>=1). All but three <i>gfbA</i>-positive WI/NI strains were erythromycin-susceptible. The remaining 33 strains, all <i>gfbA</i>-negative and WI/NI, belonged to 14 <i>emm</i> types. Twelve (36.4%) <i>gfbA</i>-negative WI/NI strains were erythromycin-resistant, all carrying <i>erm</i> genes.

In some cases, invasiveness properties seemed to be specifically correlated with the <i>emm</i> subtype. For example, <i>emm</i> subtypes stG6, stC36 and stG6792 differed between <i>gfbA</i>-positive WI/NI and HI strains. Similarly, stG6 and stG480 <i>emm</i> subtypes encountered within <i>gfbA</i>-negative WI/NI and HI strains were different, except for <i>stG480.0</i> (Fig. 2b, Table 1 and Table S1).

Recurrent infections

In four cases SDSE was recovered from the same patient; three of these strains belonged to the same clone. In particular, one case (in a child) involved persistent throat colonization by the same <i>emm</i> stG6.7 strain despite antibiotic therapy, and two cases were infections caused by <i>emm</i> stC6979.0 and stG643.0 strains, in elderly patients with underlying medical conditions. In one case, the clinical symptoms worsened over time. Additional virulence characteristics of these persistent SDSE strains were maintained over time (Table 2 and Table S1). Once only, the isolation of SDSE from the same patient 3 years apart produced different clones.

DISCUSSION

Streptococcal disease is not notifiable in Europe. Unfortunately, the European Centre for Prevention and Control (ECDC) has not included streptococcal disease (except for pneumococcus) in specific surveillance programmes or networks. Notification of streptococcal disease is, therefore, only on a voluntary basis in almost all European countries. In view of this, working with information on the genetic population structure and

![Fig. 2. Invasiveness properties and presence of the gfbA gene in relation to emm types (a) or emm subtypes (b) of SDSE strains. Black bars, gfbA-positive, HI; grey bars, gfbA-positive, WI/NI; white bars, gfbA-negative, WI/NI.](image)
virulence properties of SDSE from different sources in a European country is not a routine matter; such information is very difficult to collect and monitor over time. This study aimed to investigate the molecular epidemiology of SDSE isolates, all belonging to either Lancefield group C or G, recovered from healthy individuals and from invasive and non-invasive diseases in Italy, collected over a 10-year time frame on a voluntary basis. No difference in the properties of the bacterial strains with respect to the geographical distribution was noted. The \textit{emm} gene was successfully amplified and sequenced in all 54 SDSE isolates examined, with 18 different \textit{emm} types found. Almost all of the most frequent \textit{emm} types (\textit{stG6}, \textit{stC6979}, \textit{stG480}, \textit{stG62647}, \textit{stG6792}, \textit{stC839}, \textit{stG485}, \textit{stG643} and \textit{stC36}, in decreasing order) have also been encountered elsewhere, although with different frequencies, as reported by population genetic studies on SDSE in Australia, Europe and North America (McMillan \textit{et al.}, 2010), reflecting the diffusion of a few successful \textit{emm} types fit to disseminate in humans.

Of interest is the question of whether an association exists between specific \textit{emm} types/clones and their propensity to cause certain diseases. For GAS, specific \textit{emm} patterns have been described to correlate with tissue tropism and disease presentation (Bessen \textit{et al.}, 2000). In the case of SDSE infections, the answer is still controversial. With the proviso that the small sample size of our collection does not allow any statistical association between \textit{emm} type and specific clinical manifestation to be inferred, we cannot confirm some strong associations reported elsewhere (i.e. \textit{stG485}, \textit{stG480} and \textit{stG6} and invasive infections) (Jensen & Kilian, 2012). For example, while our findings are in agreement with the reported association of \textit{stC839} with non-invasive infections (Pinho \textit{et al.}, 2006), all nine \textit{stG6} strains here examined were isolated from either carriers or non-invasive infections.

Multi-locus sequence typing analysis has also been used to study the evolutionary relationships in SDSE, demonstrating the diffusion of few genetic lineages worldwide and the presence of lateral gene transfer and recombination events, including the core genome, in shaping the genetic variability of the SDSE population (McMillan \textit{et al.}, 2010). Nevertheless, the coupling of \textit{emm} typing with PFGE analyses of our SDSE collection gave congruent and robust results and demonstrated a satisfactory power of resolution for a real-time snapshot of the circulating clones. Generally a PFGE group was observed to cluster strains possessing exclusively the same \textit{emm} type/subtype (\textit{stG480}/PFGE group 4, \textit{stG6}/PFGE group 7.2, \textit{stG6792}/PFGE group 7.1, \textit{stC839}/PFGE group 5, \textit{stG6792}/PFGE group 16, \textit{stG6}/PFGE group 13, \textit{stC36.4}/PFGE group 12) or mostly the same \textit{emm} type (\textit{stG6792}/PFGE group 1, \textit{stG62647}/PFGE group 2), indicating the persistence of successful clones over time. Three cases of \textit{emm} types found in more than one PFGE group could also be detected; in particular, \textit{stG643} was noted to show the highest rate of genetic diversity, with three strains showing

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>\textit{emm} type</th>
<th>Time to recurrence (months)</th>
<th>Clinical signs</th>
<th>Antibiotic resistance profile</th>
<th>Virulence profile</th>
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<tr>
<td>1</td>
<td>\textit{stG6.7}</td>
<td>13</td>
<td>None</td>
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<td>4.2</td>
<td>Ulcer</td>
<td>\textit{gfbA}</td>
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<td>Bacteraemia</td>
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<tr>
<td>4</td>
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<td>9</td>
<td>Ulcer</td>
<td>\textit{mef}(E), \textit{tet}(M)</td>
<td>–</td>
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<table>
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<tr>
<td>\textit{stG480}</td>
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<tr>
<td>\textit{stG643}</td>
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\*Only at the first isolation.
three unrelated PFGE types. The high promiscuity of 
stG643 has also been described in a recent study, where this emm type was noted to be associated with five different genetic lineages (McMillan et al., 2010). A likely hypothesis is that the stG643 emm gene is located in a mobile genetic element.

In addition, a clonal cluster composed by emm stC6979/ PFGE type 16 SDSE was isolated from patients admitted to the same hospital over a 1-year period, indicating the local diffusion of a single SDSE clone. One case within this cluster involved a recurrent infection, where samples of the same clone were isolated from the same patient 1 year apart. In total, three cases of recurrent infections caused by the same SDSE clone were identified. In one case, the ability to efficiently internalize into human epithelial cells could explain the persistent throat colonization despite antibiotic therapy. This property could also be considered the mechanism responsible for other cases of recurrent bacteraemia caused by SDSE, described elsewhere (Rantala et al., 2010; Rohde et al., 2012).

At present, the complete genome sequences of three Lancefield group G and one group C SDSE (Shimomura et al., 2011; Suzuki et al., 2011; Okumura et al., 2012; Watanabe et al., 2013) plus one Lancefield group A SDSE (unpublished) are available, enabling genome-wide studies in which a GAS-to-SDSE gene flow direction has been proposed, involving the replacement of more than four additive transfers (Choi et al., 2012).

One example of lateral gene transfer acquisition by SDSE is the presence of members of a surface protein family (alpha-like proteins) (Creti et al., 2008). The Alp family comprises surface-localized protein antigens involved in adhesion and translocation of GBS across the epithelium, facilitating invasive infections. Indeed, until recently, the alpha-like proteins were supposed to be confined to GBS but, more recently, the surface protein R28, virtually identical to a member of the GBS Alp family, has been identified in S. pyogenes clinical strains. The same protein, R28 has also been detected in four unrelated strains of our collection and a new member of the Alp family, Dysalp, never to our knowledge described in GBS or GAS, has been identified (Creti et al., 2007b). In GAS, the R28 gene is included in a 37.2 kb pathogenicity island (RD2 element) (Sirkiewicz et al., 2011). Preliminary studies indicated that the same element is present in SDSE (data not shown). Our finding supports the indication of a dynamic and underestimated aspect of streptococcal pathogenesis and focuses new attention on the possible emergence of virulent clones in low-pathogenic streptococci through successive acquisitions of exogenous virulence factors.

All SDSE sequenced strains, except the Lancefield group C SDSE strain, possess clustered, regularly interspaced short palindromic repeats (CRISPR)-Cas systems that mediate resistance to infection by foreign DNA, in particular from plasmids and phages (Makarova et al., 2011; Shimomura et al., 2011; Watanabe et al., 2013). The mean number of CRISPR spacers (an indication of immunity) in SDSE was higher than in GAS (about 20 vs 4, respectively) suggesting that prophage infection of SDSE is somewhat restricted, resulting in a smaller number of virulence factors, as for example prophagic superantigens. Indeed, according to previous reports (Shimomura et al., 2011; Okumura et al., 2012), GAS superantigen genes such as speA, speH, speJ, speL, speM, ssa and smeZ were not detected in our SDSE strains, with the exception of the chromosomal speG gene, which was present in 35.2% of cases. As already observed, there was no association between clinical presentation and the presence of the speG gene (Korem et al., 2013).

No association between the ability to enter human respiratory cells at high efficiency and macrolide resistance was observed, as previously demonstrated for GAS (Facinelli et al., 2001), nor with the source of infection, and the fact that the only stG480 gfbA-positive strain is also HI, confirms the important role of the gfbA gene in cell invasion by SDSE. However, the unexpected detection of gfbA/sfbI in some WI/NI strains suggests that the presence of this invasin is not always sufficient for cell penetration and warrants further studies.

About one-third of the SDSE isolates were found to be erythromycin-resistant, as recently reported in the USA (Broyles et al., 2009). Both erm and mef genes were detected, erm(A) being the most prevalent. The erm(T) gene, recently described in S. pyogenes and S. agalactiae (DiPersio et al., 2011), was also detected, as well as mef(A) and mef(E), frequently reported in S. pyogenes and in Streptococcus pneumoniae, respectively (Varaldo et al., 2009). The erm(T) isolate has recently been characterized and found to harbour a small erm(T)-carrying plasmid mobilizable by a co-resident integrative conjugative element (ICEsde5580) to major streptococcal pathogens, including S. pyogenes, thus providing a possible explanation for the recent spread of erm(T)-carrying plasmids in streptococci (Palmieri et al., 2013). Most of the erythromycin-resistant strains were also resistant to clindamycin (phenotype MLSB); of these, >50% showed the ambiguous fMLSβ phenotype (heteroresistance to clindamycin), previously described in Korean Staphylococcus aureus isolates with constitutive and inducible expression of erm(A) (Yoon et al., 2008).

In conclusion, a molecular epidemiology investigation on SDSE recovered from different human sources was performed, we believe for the first time, in Italy and it indicates that the emm type can be considered a useful genetic marker for epidemiological studies involving SDSE, with certain emm types being largely predictive of clonal type. The indication of the acquisition of new virulence traits, as well as of rare macrolide resistance determinants by SDSE, underlines the necessity of a more comprehensive evaluation of the current SDSE infections burden in Italy and, more generally, in Europe, to keep this emerging pathogen under surveillance.
Indeed, the possibility of acquiring genetic material may lead to both an increased virulence of SDSE and the creation of a possible new reservoir for virulence/resistance genes.

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