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MOLECULAR CHARACTERIZATION OF *STREPTOCOCCUS PYOGENES* ISOLATES ASSOCIATED WITH INVASIVE INFECTIONS AND PHARYNGITIS IN PORTUGAL: EVALUATION OF TYPING METHODS AND RELATIONSHIP WITH INVASIVE ABILITY

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SUMMARY

Keywords: *Streptococcus pyogenes*, invasive infection, superantigens, molecular typing

Streptococcus pyogenes (Group A *Streptococcus*, GAS) is one of the most common human pathogens, causing mostly superficial, mild infections of the upper respiratory tract and skin, but also a variety of invasive infections that are characterized by an extremely rapid onset and progression of severe systemic signs, resulting in high morbidity and mortality. Besides characterizing the main GAS lineages causing invasive infections in different world regions, some studies have attempted to identify GAS clones with enhanced virulence that may have prompted the reemergence of invasive GAS disease witnessed by developed countries since the late 1980s. These studies have generated contrasting results, with some reporting a similar clonal composition between the GAS populations causing invasive and non-invasive infections, while others argue for the existence of particularly virulent clones that are overrepresented among invasive isolates. However, many of those studies were limited in the genetic diversity of the GAS collections analyzed and in the typing methodologies used, relying essentially on M/*emm* typing.

With the aim of characterizing the population structure and dynamics of the GAS isolates causing invasive infections in Portugal, a total of 351 isolates collected from normally sterile sites throughout the country between 2000 and 2009 were studied by *emm* typing, T typing, pulsed-field gel electrophoresis (PFGE) macrorestriction profiling, superantigen (SAg) gene profiling, multilocus sequence typing (MLST), and antimicrobial susceptibility testing. In order to evaluate the presence of GAS clones with enhanced invasive ability and to identify potential genetic markers of invasiveness, the properties of the 160 invasive isolates collected during 2000-2005 in Portugal were compared with those of a collection of 360 isolates associated with pharyngitis, recovered during the same time period.

The GAS isolates causing invasive infections in Portugal presented a high genetic diversity, despite the dominance of a PFGE-defined lineage of macrolide-susceptible isolates presenting *emm*1-T1-ST28 and the SAg genes *speA*, *speG*, *speJ*, and *smeZ*, which is widely disseminated in developed regions and significantly associated with invasive infections in Portugal and other countries. Another PFGE cluster was significantly overrepresented among invasive isolates in Portugal, when compared with the isolates causing pharyngitis, composed mainly of isolates of *emm*64-ST164 and carrying the *speG* and *smeZ* genes. This cluster, in

contrast to the *emm*1-T1-ST28 lineage, has not been frequently identified among invasive isolates in other countries and presented a very low prevalence in the period of 2006-2009 in Portugal.

Specific individual characteristics, namely *emm*1, *emm*64, and the presence of *speA* or *speJ* genes were identified as genetic markers of invasiveness, while *emm*4, *emm*75, and the *ssa*, *speL*, and *speM* genes were associated with a decreased ability to cause invasive infections.

The overall clonal distribution of the isolates causing invasive GAS infections in Portugal showed significant fluctuations between the two time periods of the study (2000-2005 and 2006-2009), in agreement with recent observations from other developed countries. Interestingly, in spite of an overall decrease in the diversity of the circulating invasive GAS lineages in Portugal, a genetic diversification of some of these lineages was observed. This was reflected by a higher diversity of SAg profiles within these clones, in the period of 2006-2009, due to loss and acquisition of SAg genes, as well as by the occurrence of macrolide-resistant isolates in clones that were previously uniformly susceptible, suggesting a recent acquisition of resistance determinants.

Macrolide resistance was significantly lower among the isolates recovered from invasive infections (12%) than among those associated with pharyngitis (21%) ($P = 0.016$), in contrast to tetracycline resistance, which was higher in the invasive isolates (17% vs 6% in pharyngitis isolates, $P < 0.001$). Remarkably, among the isolates sharing *emm*1-T1-ST28 and *emm*4-T4-ST36, only the PFGE clusters grouping macrolide-susceptible isolates were significantly associated with invasive infection or pharyngitis, respectively, while the macrolide-resistant clones presented a similar distribution among the two infection types. The clonal spread of isolates with reduced fluoroquinolone susceptibility reported in other countries was not observed among invasive infections in Portugal, despite the identification of two isolates with reduced susceptibility and one with high level resistance to levofloxacin.

The comparison of the several typing methods used regarding to their concordance and discriminatory power, based on a genetically diverse collection of GAS isolates, confirmed previous findings showing that *emm* typing is not sufficient for an accurate discrimination of GAS clones, requiring complementation with other typing methods. PFGE was generally the most efficient in predicting the other molecular properties and was the only method capable of discriminating isolates of the same *emm* type according to macrolide resistance and disease presentation. SAg gene profiling was able to further discriminate

isolates sharing the same *emm* type, reflecting a shorter time-scale of genetic evolution than the remaining molecular typing methods.

The SAg screening of the isolates was performed by two multiplex polymerase chain reactions (PCR) using as positive control fragments the chromosomal genes *speB* and *speF*, which encode a cysteine protease and a deoxyribonuclease, respectively. Following the identification of four isolates lacking one or both of these genes, four distinct deletions of different extensions in the chromosomal region comprising *speB*, *speF* and the transcriptional regulator gene *rgg* were characterized in detail.

The studies presented in this thesis provide the first detailed molecular characterization of the GAS isolates causing invasive infections in Portugal, highlighting the prevalence of widely disseminated lineages, but also the local emergence of invasive clones with limited geographic and temporal expansion. The results emphasize the importance of using multiple typing methods in order to achieve an unambiguous discrimination of GAS isolates, and indicate an ongoing evolution of the genetic lineages causing invasive infections in Portugal, associated with horizontal gene transfer mechanisms. The identification of several genetic markers linked to either increased or decreased invasive potential underscores the role of bacterial genetic factors for the outcome of GAS infections. However, the molecular properties of the majority of the isolates causing invasive infections in Portugal reflected the clonal structure presented by the pharyngitis-causing GAS population, suggesting that other, yet unidentified factors must also play an important role in disease presentation.

RESUMO

Palavras-chave: *Streptococcus pyogenes*, infecção invasiva, superantigénios, tipagem molecular

Streptococcus pyogenes (*Streptococcus* do Grupo A de Lancefield, GAS) é um dos agentes patogénicos mais comuns no ser humano, sendo responsável sobretudo por infecções superficiais ligeiras, mas podendo também causar uma variedade de infecções invasivas que se caracterizam por um aparecimento e progressão extremamente rápidos de manifestações sistémicas graves, resultando em elevados níveis de morbilidade e mortalidade. Para além de procederem à caracterização das principais linhagens de GAS associadas a infecção invasiva em diversas regiões geográficas, alguns estudos procuraram identificar clones de GAS com virulência aumentada, que possam estar na origem do reaparecimento das infecções invasivas registado nos países desenvolvidos desde os finais da década de 1980. Estes estudos originaram resultados contraditórios, alguns dos quais sugerem uma composição clonal semelhante entre populações de GAS associadas a infecções invasivas e não invasivas, enquanto outros evidenciam a existência de clones particularmente virulentos que se encontram sobre-representados nas infecções invasivas. No entanto, muitos destes estudos são limitados ao nível da diversidade genética dos conjuntos de estirpes[*] analisados, bem como das metodologias de tipagem utilizadas, baseando-se sobretudo na tipagem M/*emm*.

Com o intuito de caracterizar a estrutura e dinâmica da população de GAS causadora de infecções invasivas em Portugal, 351 estirpes isoladas de locais habitualmente estéreis, entre 2000 e 2009, em diversas regiões do país, foram estudadas através de um conjunto alargado de métodos utilizados em estudos epidemiológicos de GAS, nomeadamente tipagem *emm*, tipagem T, análise dos perfis de macro-restrição do DNA por electroforese em campo pulsado (PFGE, "Pulsed-Field Gel Electrophoresis Profiling"), determinação do perfil de genes de superantigénios (SAgs), "Multilocus Sequence Typing" (MLST) e testes de susceptibilidade a antimicrobianos. De forma a avaliar a presença de clones de GAS com capacidade invasiva aumentada e identificar potenciais marcadores genéticos de invasibilidade, as propriedades das 160 estirpes invasivas isoladas entre 2000 e 2005 foram comparadas com as de um conjunto de 360 estirpes associadas a faringite, obtidas durante o mesmo período, em Portugal.

[*] Neste trabalho, a palavra estirpe é utilizada para referir um microrganismo isolado num determinado produto biológico.

As estirpes de GAS associadas a infecção invasiva em Portugal apresentaram uma elevada diversidade genética, apesar do predomínio de um clone de PFGE constituído por estirpes susceptíveis aos macrólidos, caracterizadas por *emm*1-T1-ST28 e contendo os genes *speA*, *speG*, *speJ* e *smeZ*. Este clone encontra-se disseminado a nível mundial e foi significativamente associado a infecção invasiva, tanto em Portugal como noutros países. Identificou-se um outro agrupamento de PFGE significativamente sobre-representado em estirpes invasivas em Portugal, por comparação com as de faringite, constituído principalmente por estirpes de *emm*64-ST164 contendo os genes *speG* e *smeZ*. Este clone, contrariamente à linhagem *emm*1-T1-ST28, não tem sido identificado com frequência em estirpes invasivas noutros países e apresentou uma prevalência bastante reduzida no período de 2006-2009 em Portugal.

Foram identificadas características individuais específicas, nomeadamente *emm*1, *emm*64, e a presença dos genes *speA* ou *speJ*, como marcadores genéticos de invasibilidade, enquanto os tipos *emm*4 e *emm*75, bem como os genes *ssa*, *speL* e *speM* foram associados a uma capacidade diminuída de provocar infecção invasiva.

A distribuição global dos clones de estirpes associadas a infecções invasivas em Portugal sofreu flutuações significativas entre os dois períodos do estudo (2000-2005 e 2006-2009), em consonância com observações recentes de outros países desenvolvidos. Apesar de se ter registado uma diminuição global da diversidade de linhagens de GAS responsáveis por infecções invasivas a circular em Portugal, observou-se um aumento da diversidade genética de algumas delas. Este facto traduziu-se por uma maior diversidade de perfis de SAgs nestes clones, no período de 2006-2009, devido a aquisição e perda de genes de SAgs, bem como pelo aparecimento de estirpes resistentes aos macrólidos em clones que no período anterior eram uniformemente susceptíveis, sugerindo uma aquisição recente de determinantes genéticos de resistência.

A resistência aos macrólidos foi significativamente mais baixa entre as estirpes isoladas de infecções invasivas (12%) do que entre as de faringite (21%) ($P = 0.016$), por oposição à resistência à tetraciclina, que foi superior nas estirpes invasivas (17% vs 6% nas estirpes de faringite, $P < 0.001$). É de salientar que, de entre as estirpes caracterizadas por *emm*1-T1-ST28 e por *emm*4-T4-ST36, apenas se identificou associação significativa com infecção invasiva ou faringite, respectivamente, para os clones de PFGE que agruparam as estirpes susceptíveis aos macrólidos, enquanto os clones de estirpes resistentes com as mesmas características moleculares apresentaram uma distribuição semelhante entre os dois tipos de infecção. A expansão clonal de estirpes com susceptibilidade reduzida às

fluoroquinolonas que tem sido descrita noutros países não foi observada em infecções invasivas em Portugal, apesar da identificação de duas estirpes com susceptibilidade reduzida e de uma com elevada resistência à levofloxacina.

A comparação dos vários métodos de tipagem utilizados no que diz respeito à sua concordância e poder discriminatório, baseada num conjunto de estirpes de GAS com elevada diversidade genética, permitiu confirmar resultados anteriores que demonstravam que a tipagem *emm* não é suficiente para uma correcta discriminação dos clones de GAS, devendo ser complementada com outros métodos de tipagem. Na generalidade, a análise por PFGE foi o método mais eficiente a prever as restantes propriedades moleculares e foi o único método capaz de discriminar estirpes do mesmo tipo *emm* de acordo com a resistência aos macrólidos e tipo de infecção. A determinação do perfil de SAgs permitiu discriminar estirpes com o mesmo tipo *emm*, reflectindo uma escala de tempo evolutivo mais curta do que os restantes métodos de tipagem analisados.

A determinação dos perfis de SAgs foi efectuada através de duas reacções de polimerização em cadeia (PCR, "Polymerase Chain Reaction"), utilizando como fragmentos de controlo positivo os genes cromossómicos *speB* e *speF*, que codificam, respectivamente, uma protease de cisteínas e uma desoxirribonuclease. Subsequentemente à identificação de quatro estirpes que não apresentavam um ou nenhum destes dois genes, foram caracterizadas quatro delecções de diferentes extensões na região cromossómica que inclui os genes *speB*, *speF* e também o gene do regulador transcripcional *rgg*.

Os estudos descritos na presente tese constituem a primeira caracterização molecular detalhada das estirpes de GAS associadas a infecção invasiva em Portugal, salientando a prevalência de linhagens amplamente disseminadas, mas também a emergência local de clones invasivos mais limitados a nível geográfico e temporal. Os resultados sublinham a importância da utilização de múltiplos métodos de tipagem com vista a uma correcta discriminação das estirpes de GAS e sugerem estar em curso a evolução de algumas das linhagens responsáveis por infecções invasivas em Portugal, associada a mecanismos de transferência genética horizontal. A identificação de vários marcadores genéticos indicadores de um potencial invasivo aumentado ou reduzido salienta o papel dos factores genéticos bacterianos no resultado das infecções por GAS. Contudo, as propriedades moleculares da maioria das estirpes isoladas de infecções invasivas em Portugal refletem a composição clonal da subpopulação de GAS associada a faringite, sugerindo que outros factores ainda não identificados poderão também desempenhar um importante papel na manifestação clínica da infecção.

THESIS OUTLINE

The present thesis describes the epidemiological and molecular characterization of the GAS population causing invasive infections in Portugal throughout the 2000's decade, as well as its comparison with the clonal structure of the GAS isolates causing pharyngitis, using a variety of phenotypic and genotypic typing methods.

In **Chapter I**, the issues addressed throughout the thesis are put into context, by providing an overview of the clinical relevance of *Streptococcus pyogenes* and reviewing important aspects of the global GAS epidemiology, population structure and evolution, with particular emphasis on invasive infections. The contribution of different virulence factors to GAS pathogenesis, the most commonly used phenotypic and molecular methods for the epidemiological typing of this species, and the impact of GAS molecular epidemiology and antimicrobial resistance for the management of GAS infections are also addressed. The aims of the thesis are presented in detail at the end of this chapter.

Chapter II describes the clonal structure of the GAS population causing invasive infections in Portugal between 2000 and 2005 and evaluates the geographic dissemination of the main lineages using multilocus sequence typing.

Chapter III presents a comparison between the molecular properties and antimicrobial resistance of the invasive GAS isolates characterized in Chapter II and those of a collection of isolates recovered from patients with pharyngitis in Portugal, during the same time period. The results of this comparison highlight the circulation of clones with an enhanced ability to cause invasive infection and identified molecular markers of increased and decreased invasiveness.

In **Chapter IV**, the SAg gene content of all isolates studied in chapters II and III is analyzed in detail by a novel multiplex PCR scheme. A comparison between all the molecular typing methods used in this thesis is performed and several significant associations between SAg genes or between SAg genes and other molecular properties are described. The results provide insights into the relevance of the SAg gene content of *S. pyogenes* for the epidemiological typing and the genetic evolution of this pathogen. Additionally, four naturally occurring deletions involving the chromosomal region that comprises the genes *speB*, *speF*, and *rgg* are characterized.

In **Chapter V**, an update of the molecular epidemiology of invasive GAS infections in Portugal is provided by the characterization of the invasive isolates recovered between 2006

and 2009 throughout the country. The results are compared with those obtained in the previously studied time period (described in Chapters II and III), highlighting a genetic diversification of some of the prevalent genetic lineages.

Finally, **Chapter VI** presents an integrated overview of the major findings described in the previous chapters, indicating some questions that remain unanswered and that can be the basis for future research.

Chapters II-V are reproductions of the following publications:

Chapter II: Friães, A., M. Ramirez, J. Melo-Cristino, and the Portuguese Group for the Study of Streptococcal Infections. 2007. Nonoutbreak surveillance of group A streptococci causing invasive disease in Portugal identified internationally disseminated clones among members of a genetically heterogeneous population. J. Clin. Microbiol. **45**:2044–2047.

Chapter III: Friães, A., F. R. Pinto, C. Silva-Costa, M. Ramirez, and J. Melo-Cristino. 2012. Group A streptococci clones associated with invasive infections and pharyngitis in Portugal present differences in *emm* types, superantigen gene content and antimicrobial resistance. BMC Microbiol. **12:**280.

Chapter IV: Friães, A., F. R. Pinto, C. Silva-Costa, M. Ramirez, and J. Melo-Cristino. 2013. Superantigen gene complement of *Streptococcus pyogenes*-relationship with other typing methods and short-term stability. Eur. J. Clin. Microbiol. Infect. Dis. **32**:115–125.

Chapter V: Friães, A., J. P. Lopes, J. Melo-Cristino, M. Ramirez, and the Portuguese Group for the Study of Streptococcal Infections. 2013. Changes in *Streptococcus pyogenes* causing invasive disease in Portugal: evidence for superantigen gene loss and acquisition. (Submitted to International Journal of Medical Microbiology)

ABBREVIATIONS

ADP	Adenosine diphosphate
AW	Adjusted Wallace coefficient
C4BP	C4-binding protein
CDC	Centers for disease control and prevention
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
DLV	Double-locus variant
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECM	Extracellular matrix
FCT	Fibronectin-binding, collagen-binding, T antigen
FDR	False discovery rate
FHL	Factor H-like
GAS	Group A streptococci
HLA	Human leukocyte antigen
IFN	Interferon
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
M	Macrolides (resistance phenotype)
MGE	Mobile genetic element
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLS_B	Macrolides, lincosamides, streptogramins B (resistance phenotype)
$cMLS_B$	constitutive MLS_B resistance phenotype
$iMLS_B$	inducible MLS_B resistance phenotype
MLST	Multilocus sequence typing
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADase	NAD glycohydrolase
NET	Neutrophil extracellular trap
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMN	Polymorphonuclear leukocyte

PYR	Pyrrolidonylarylamidase
QRDR	Quinolone resistance-determining region
RALP	RofA-like protein
rRNA	ribosomal ribonucleic acid
SAg	Superantigen
Sic	Streptococcal inhibitor of complement
SID	Simpson's index of diversity
SLO	Streptolysin O
SLS	Streptolysin S
SLV	Single-locus variant
SMEZ	Streptococcal mitogenic exotoxin Z
SOF	Serum opacity factor
Spe	Streptococcal pyrogenic exotoxin
SSA	Streptococcal superantigen
ST	Sequence type
STSS	Streptococcal toxic shock syndrome
TCR	T cell receptor
TCS	Two-component system regulator
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UPGMA	Unweighted pair group method with arithmetic mean
W	Wallace coefficient
WGS	Whole-genome sequencing

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CHAPTER I

GENERAL INTRODUCTION

1. Historical overview

The designation "*Streptococcus pyogenes*" was first used by Friedrich Rosenbach in 1884 to name a bacterial strain associated with wound infections. Strains isolated from infections like erysipelas, puerperal fever, and scarlet fever, and originally assigned with distinct names (*Streptococcus erysipelatis*, *Streptococcus puerperalis*, *Micrococcus scarlatinae*, *Streptococcus scarlatinae*, *Streptococcus hemolyticus*), were later acknowledged as belonging to the same species than Rosenbach's strain [49] (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). In 1933, Rebecca Lancefield proposed a scheme for the serologic classification of hemolytic streptococci based on the precipitation of the group-specific cell wall carbohydrate [100] and *Streptococcus pyogenes* became also known as Group A *Streptococcus* (GAS).

Before World War II, this species was mostly known for causing severe puerperal infections, scarlet fever, and for being a major cause of death in burn patients, but the introduction of penicillin was responsible for a marked decline in the incidence of these infections in developed countries. However, since the 1980s, a resurgence of severe invasive GAS disease, both supurative infections and non-supurative sequelae, was reported in several industrialized regions. Currently, in developed countries *S. pyogenes* is well known for causing mild superficial infections of the upper respiratory tract and skin, like sore throat and impetigo, but also severe and rapidly progressing invasive infections, like necrotizing fasciitis, which made this organism popularly known as the "flesh-eating" bacterium due to the extensive necrosis of deep soft tissue [33, 44].

2. General features and identification of *S. pyogenes*

Streptococcus pyogenes are Gram-positive, nonmotile, and nonsporeforming spherical cocci with 0.5 to 1.0 µm, which can form long chains in liquid medium, although they occur in short chains or pairs in clinical specimen [136]. *S. pyogenes* cells are covered with a capsule of hyaluronic acid, a high molecular weight polymer of alternating residues of N-acetylglucosamine and glucoronic acid [14]. This facultative anaerobe is catalase negative, requiring a catalase source for growth in aerobic conditions, usually blood- or serum-enriched medium [136]. Despite being a free-living organism, the only ecological niche known for *S. pyogenes* is the human host, where it can cause infection or establish an asymptomatic

colonization of the nasal and oropharyngeal mucosal epithelium and, transiently, of the superficial layers of the epidermis (section 3). Therefore, transmission is believed to be mostly from person to person [10].

The hemolysis pattern on 5% blood agar plates is used as a classification scheme for streptococci, with three distinct patterns recognized: α-hemolysis (partial or green hemolysis), β-hemolysis (complete hemolysis), and γ-hemolysis (absence of hemolysis). *S. pyogenes* is capable of producing complete hemolysis even during aerobic growth, due to the secretion of two hemolysins, namely streptolysin-O (SLO, which is inhibited by oxygen) and streptolysin-S (SLS, which is not affected by oxygen). Hence, after 24 hours of incubation on blood agar at 35-37ºC, *S. pyogenes* isolates typically exhibit 1-2 mm whitish colonies with large zones of β-hemolysis (Figure I-1), although larger colonies with a mucoid appearance may be observed in strains with increased expression of the hyaluronic acid capsule [136]. There are, however, reports of *S. pyogenes* strains presenting α- or γ-hemolysis on blood-agar, most likely due to the genetic inactivation of the SLS-encoding *sag* operon [215].

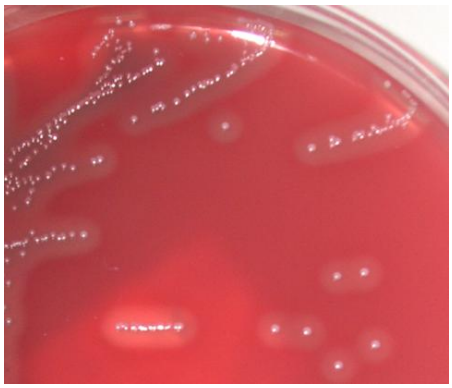

Figure I-1 *S. pyogenes* grown for 24h at 35ºC on sheep blood-agar. The whitish colonies present the typical β-hemolysis zones.

Following the presumptive identification based on the characteristic aspect of the colonies on blood agar, two methods are traditionally used to confirm species identification: the bacitracin susceptibility test and the PYR test. The latter tests the isolates for pyrrolidonylarylamidase activity. *S. pyogenes* is the only β-hemolytic *Streptococcus* positive for both tests, although none of them alone is 100% species specific [50]. Significant fractions of *S. pyogenes* isolates exhibiting bacitracin resistance have been identified in Portugal and other countries [117, 159, 179], and therefore the bacitracin susceptibility test is no longer considered a reliable method for the identification of this species.

A more reliable, fast and easy to perform alternative is a latex agglutination test based on the serological grouping system developed by Rebecca Lancefield [100], which is based on a specific antigen, in most cases a cell wall carbohydrate, present in most β-hemolytic and

some α- and γ-hemolytic streptococci. In the case of *S. pyogenes*, the cell-wall carbohydrate is a dimer of *N*-acetylglucosamine and rhamnose [136]. After the enzymatic extraction of this carbohydrate, *S. pyogenes* isolates agglutinate only with the group A specific serum. The same group-specific carbohydrate can be found in a few strains of *Streptococcus dysgalactiae* subsp. *equisimilis* and of the *Streptococcus anginosus* group. However, since globally these are less frequently isolated from human infections, the term "GAS" is commonly used as a synonym for *S. pyogenes* [50]. Differentiation of this organism from other group A species can usually be achieved simply by the PYR test.

3. GAS infections

S. pyogenes is one of the most common human pathogens and is able to cause a wide range of infections, which may occur endemically or as epidemics. The upper respiratory tract and skin are the main reservoirs for GAS transmission and also the primary focal sites of infection, where superficial, uncomplicated diseases can develop. However, *S. pyogenes* can also disseminate into deeper tissues and cause severe invasive infections that are associated with high morbidity and mortality. Serious systemic symptoms frequently arise in response to the production of bacterial toxins and immunologically mediated non-suppurative sequelae may develop following streptococcal infections [33, 44].

3.1. Colonization

Although *S. pyogenes* is not considered as part of the normal human pharyngeal flora, colonization of the oropharynx by GAS can result in a long-term carrier state, characterized by a lack of clinical symptoms and of a vigorous immune response [10, 33]. A GAS carrier is usually defined as an asymptomatic individual with a positive throat culture and no serologic response, or with a positive throat culture after completion of an appropriate antimicrobial treatment [121]. However, the studies evaluating the prevalence of GAS carriage usually do not test for serologic markers of infection and rely mainly on the absence of clinical symptoms for the definition of the carrier state. Carriage prevalence is highly variable, depending on factors like the geographic location, the studied population, and the methods used in the study. The highest carriage rates are observed in school-age children [71, 173]. A recent meta-analysis including studies from several countries estimated an average

colonization rate of 12% [95% confidence interval (CI) 9% to 14%] among children [173], while in adults, rates of 2-3.7% have been reported [37, 71, 157]. In the Lisbon area, the asymptomatic GAS colonization rate has been estimated to be 10.7% among children and 3.3% among household adults [157], values in line with the meta-analysis estimates.

Since the organism remains capable of transmission to a new host during the carrier state, oropharyngeal carriage in children is considered an important GAS reservoir that may facilitate the spread of the bacterium and the emergence of outbreaks, especially within crowded settings like schools and day-care centers, as well as among household contacts [10, 43]. In Minnesota, U.S.A., a community outbreak of invasive GAS infections has been associated with high rates of pharyngeal carriage of GAS isolates with a high invasive potential among school children [26].

Several mechanisms have been proposed to be involved in the establishment of the carrier state, including the occurrence of mutations that downregulate virulence, the production of a host immune response that limits pathogen proliferation, and the internalization of GAS into host cells, enabling the persistence of the pathogen in an environment protected from phagocytes, humoral immunity and antibiotics that do not efficiently cross the eukaryotic membrane, like penicillin [14, 194].

In contrast to oropharyngeal carriage, colonization of the skin by *S. pyogenes* is apparently transient and is not commonly associated with GAS transmission [10]. However, the short-term colonization of the skin may constitute the first step in GAS skin and soft tissue infections, in which the colonizing organism may gain access to deeper tissues through a penetrating injury [136], as explained below.

3.2. Suppurative infections

Tonsillo-pharyngitis is the most common infection caused by GAS in temperate climate regions, affecting mostly school-age children (5-15 years of age), especially during late fall and winter. This pathogen is estimated to cause 20-30% of pharyngitis cases in children and 5-15% of cases in adults, being the leading bacterial agent of tonsillo-pharyngitis in humans [25, 175]. *S. pyogenes* pharyngitis, commonly known as “strep throat”, is characterized by a sudden onset of sore throat, pain on swallowing, and fever. Tonsillopharyngeal erythema is observed, often with a patchy, grayish-yellow exudate and with lymphadenitis [72, 175]. GAS pharyngeal infections, and less commonly other GAS

infections, are sometimes accompanied by scarlet fever, leading to the development of a diffuse erythematous rash [33, 136]. Since the transmission of pharyngeal infections occurs mainly through respiratory droplets, attendance of crowded settings, like nursing homes, schools, and military facilities increases the infection risk both in adults and in children [25, 33].

The other primary focal site of GAS infection is the skin. Pyoderma or impetigo is a highly contagious infection that occurs mainly in economically disadvantaged young children (2-5 years of age), especially in tropical regions or during summer in temperate climate areas [25]. It affects mostly exposed body areas (face, arms, legs) and is characterized by the development of vesicles and pustules in a confined region of the superficial skin, although spread of the infection due to scratching is common. Erysipelas is an acute infection involving more extensive skin areas than impetigo, and occurs most commonly in young children and older adults [136]. It is characterized by local inflammation, with a distinct differentiation of the infected skin, which raises above the surrounding, uninfected tissue. Cellulitis involves not only the skin, but also subcutaneous tissue and the distinction between infected and uninfected skin is not clear. In both these conditions, patients also experience systemic symptoms, like fever and leukocytosis, and bacteremia occurs in about 5% of cases [15].

Necrotizing fasciitis is a highly destructive infection involving subcutaneous and deep soft tissue, often affecting muscle, which occurs most commonly in the lower and upper extremities. Initially, patients present with an apparently benign skin lesion, often mistaken for an insect bite, and nonspecific symptoms like fever, vomiting, and diarrhea. The local lesion rapidly progresses to swelling, followed by the appearance of purple bullae filled with a clear fluid. The infection spreads within hours to a couple of days, resulting in an extensive necrosis of deep tissue, fat, and frequently muscle (myositis), due to the combined action of GAS virulence factors and tissue-damaging enzymes released by the host polymorphonuclear leukocytes. The inflammatory process is accompanied by marked systemic signs, which ultimately include shock and multiorgan failure [15, 136, 149, 189]. GAS bacteremia, which is present in most patients with necrotizing fasciitis, is often linked with streptococcal toxic shock syndrome (STSS, discussed below). Necrotizing fasciitis is associated with high mortality rates (20-50%), especially when STSS develops. In the presence of myositis, which can occur alone or, more frequently, in association with necrotizing fasciitis, mortality rates of 80-100% have been reported [189].

A definite case of STSS is defined by the isolation of GAS from a normally sterile site, associated with hypotension and two of the following clinical signs: renal impairment,

coagulopathy, liver involvement, adult respiratory distress syndrome, erythematous macular rash, soft-tissue necrosis [124]. Patients initially present with symptoms like myalgia, malaise, chills, fever, vomiting, diarrhea, swollen lymph nodes, and increasingly severe local pain in the cases associated with skin and soft tissue infections. Severeness of the symptoms rapidly increases, ultimately culminating in hypotension, shock, and multiorgan failure [15, 124]. The majority of STSS patients are also bacteremic [124].

GAS bacteremia is frequently associated with soft tissue infections, but it can also occur in association with a variety of other invasive suppurative infections, such as puerperal sepsis, pneumonia, meningitis, septic arthritis, and endocarditis, although the incidence of these infections has decreased substantially since the introduction of antimicrobial agents [44, 136]. However, bacteremia is often the only clinical diagnosis, without an evident focal infection site [99, 143].

Severe invasive GAS infections can begin locally, at the site of a preceding skin trauma that may constitute a cutaneous portal of entry for the pathogen, which may transiently colonize the skin. However, a significant fraction of patients does not report antecedent skin injury, in which case the origin of the infection is unclear [149]. Up to 50% of STSS cases do not present an identified portal of entry, although many of these patients develop a deep-seated infection like necrotizing fasciitis or myositis during the course of the disease [124, 189]. It is generally believed that in such cases GAS isolates migrate from a colonized mucous membrane, most likely the pharynx, to the infection site through the blood, creating a transient bacteremia [15, 189]. Non-penetrating injuries like blunt trauma, muscle strain, or hematoma somehow favor bacterial growth at these sites and constitute risk factors for the development of GAS invasive infections [189]. Other predisposing factors have been reported, including penetrating injuries, burns, surgical procedures, childbirth, viral infections like varicella and influenza, and the use of non-steroidal anti-inflammatory drugs [15, 149, 189]. Transmission of invasive GAS infections from person to person is not common, although some clusters of infections in nursing homes, health care workers, and family members have been reported [189].

3.3. Non-suppurative sequelae

S. pyogenes infections can be followed by immune-mediated, non-suppurative diseases. Rheumatic fever can develop 1-5 weeks after a pharyngitis episode and is characterized by an inflammatory process involving the joints (arthritis), heart (carditis), central nervous system (chorea), skin (erythema marginatum), and/or subcutaneous nodules [33]. The disease is auto-immune in nature, resulting most likely from the production of antibodies and T cells against streptococcal surface macromolecules that cross-react with host tissues. Hence, the pathogenesis of this disease involves a mechanism of molecular mimicry, i.e. the sharing of epitopes between antigens. Several streptococcal components have been suggested to be involved, but the most relevant is the M protein (section 4.1), which cross-reacts with cardiac and skeletal myosin, among other host antigens [24, 25, 33]. Rheumatic fever is a major public health concern in many developing countries with poor sanitary conditions and limited access to antibiotics, where it is considered a major cause of acquired heart disease, especially among children [21, 25]. In developed countries, the use of antimicrobial agents in the treatment of pharyngitis greatly reduced the incidence of rheumatic fever, although a resurgence of the disease has been reported in some developed regions, usually associated with specific GAS serotypes [154, 206].

Acute glomerulonephritis is another post-streptococcal non-supurative disease, which can develop 3 to 6 weeks after a GAS skin infection, or 1 to 2 weeks after a streptococcal pharyngitis [33]. It is characterized by an acute inflammation of the renal glomeruli, resulting in a sudden onset of edema of the face and extremeties, hematuria, proteinuria, hypertension, and decreased serum complement levels [33, 136]. This post-streptococcal sequel occurs mostly in children and young adults. While children usually have a full recovery, adults frequently develop chronic glomerulonephritis or hypertension. Nephritogenic GAS strains belong to specific serotypes (section 5.1) and molecular mimicry between the M protein (section 4.1) of these strains and the glomerular basement membrane has been proposed as one of the key mechanisms involved in the pathogenesis of the disease [33]. The incidence of post-streptococcal acute glomerulonephritis has been continuously declining in developed countries, but it is still high in some regions of the world, like Africa, South America, New Zealand, and India [25, 33].

Although less common, other non-suppurative diseases have been associated with GAS infections, including streptococcal reactive arthritis and brain disorders like PANDAS

(Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections) [122, 203].

4. Virulence factors

Streptococcus pyogenes has evolved a wide spectrum of both cell-associated and secreted virulence factors that promote the adaptation of the pathogen to the human host, some of them playing multiple roles in the bacterial pathogenic mechanism (Table I-1). Many of these factors have been discovered in recent years through the analysis of complete genome sequences of GAS strains, and new molecules and mechanisms contributing to GAS pathogenesis are constantly being proposed [20, 78, 109]. Although most of the virulence factors are part of the GAS core chromosome, being present in virtually all GAS strains, others are found in a limited number of GAS lineages due to their location in foreign genetic elements. Moreover, several virulence factors present allelic variation. These inter-strain differences may translate the propensity of distinct GAS clones to specific host tissues or to cause certain infection types.

It is widely accepted that the first stage of GAS pathogenesis is the establishment of host-pathogen interactions through the adherence of the bacteria to the host tissues, usually the tonsillo-pharyngeal or cutaneous epithelia [14, 28, 33]. The lipoteichoic acid present on the streptococcal cell wall has been proposed to mediate the first step of this adherence by the establishment of hydrophobic interactions that promote a close contact of the bacteria with the host cells, then allowing a high-affinity binding mediated by specific adhesins [73]. A large number of streptococcal cell-surface molecules have been reported as possible adhesins due to their ability to bind specific components of the host tissues [141, 214]. The main GAS elements involved in adhesion are presented in Table I-1, including the M protein (section 4.1) and the hyaluronic acid capsule, which are recognized as two major virulence factors of GAS due to their involvement in multiple pathogenic mechanisms. The most important host ligand involved in the attachment mechanism seems to be fibronectin, an extracellular matrix (ECM) glycoprotein that is recognized by a large family of fibronectin-binding proteins produced by GAS [214].

Table I-1 Mechanism of action of the main virulence factors of *S. pyogenes*.

Virulence factor	Function				References
	Adherence	**Dissemination**	**Immune evasion**	**Toxicity**	
Lipotheicoic acid	Hydrophobic interactions with host cells; fibronectin binding				[73]
Fibronectin-binding proteins (F1/PrtF1/SfbI, F2/PrtF2/PFBP, SfbII/SOF, SfbX, Fbp54, FbaA, FbaB)*	Fibronectin/fibrinogen binding		FbaA and PrtF1 inhibit opsonization by C3b		[214]
Collagen-, vitronectin-, and galactose-binding proteins	Binding to collagen/vitronectin/galactose				[33]
Pili	Binding to human pharyngeal epithelium and keratinocytes by unknown mechanism				[1, 119]
SlaA (phospholipase A2)*	Binding to human epithelial cells by unknown mechanism			Cytotoxic for epithelial cells	[182]
Hyaluronic acid capsule	Binding to CD44 on human epithelial cells	CD44-mediated disruption of epithelial cell-cell junctions	Physical barrier to complement-mediated phagocytic killing; inhibition of LL-37, promoting survival in NETs		[28, 213]
M protein	Fibronectin binding (not all M proteins); binding to CD46 on keratinocyte membrane; binding to glycosaminoglycans on epithelial cells and skin fibroblasts	Plasminogen binding, contributing to degradation of fibrin clots and ECM components	Binding to factor H, FHL-1, fibrinogen, and C4BP, inhibiting the classical and alternate complement pathways and phagocytosis; immunoglobulin binding	Binding of kininogen, contributing to bradykinin release; M1-fibrinogen complexes activate neutrophils; M1 interacts with TLR2 and activates T cells, inducing cytokine release	[60, 146, 183]
M-like proteins		Plasminogen binding, contributing to degradation of fibrin clots and ECM components	Immunoglobulin binding; resistance to phagocytosis		[29, 33]
Glyceraldehyde-3-phosphate dehydrogenase	Binding to fibronectin and cytoskeletal proteins	Plasminogen binding, contributing to degradation of fibrin clots and ECM components	Binds C5a, cooperating with C5a peptidase		[29, 152, 214]
Enolase		Plasminogen binding, contributing to degradation of fibrin clots and ECM components			[29]
Streptokinase		Activation of plasminogen to plasmin, promoting degradation of fibrin clots and ECM components			[29]
Hyaluronidase		Degradation of hyaluronic acid present in connective tissue			[14]

*Variable presence among GAS genetic lineages.

Table I-1 (continued) Mechanism of action of the main virulence factors of *S. pyogenes*.

Virulence factor	Function				References
	Adherence	Dissemination	Immune evasion	Toxicity	
DNases*		DNA degradation, contributing to pus liquefaction	Degradation of DNA in NETs, avoiding bacterial entrapment (in particular, DNase Sda1)		[14, 19]
SpeB (cysteine protease)		Protease activity, contributing to pus liquefaction; degradation of fibronectin and vitronectin; activation of host cell matrix metalloproteases that degrade host tissue components	Immunoglobulin cleavage; LL-37 cleavage; degradation of C3b and properdin, inhibiting complement activation; chemokine degradation	Activation of cytokine IL-1β; activation of kinins and histamines; degradation of fibrinogen may interfere with blood clotting	[87, 140]
C5a-peptidase			C5a inactivation, blocking the recruitment of phagocytes		[86]
Mac/IdeS/MspA			IgG endopeptidase activity, preventing opsonophagocytosis; CD16B binding, inhibiting PMN activation and killing		[28]
Sic*			Inhibition of the complement membrane attack complex; ezrin binding inhibits phagocytosis and killing by PMNs; inhibition of lysozyme, defensins, IFN-γ, LL-37		[56, 77, 149]
SPyCEP/ScpC			Cleavage of chemokines, inhibiting PMN recruitment and activation		[75, 192]
Streptolysin O			Damaging of macrophages and lysossomes	Pore-forming cytolysin, damaging erythrocytes and platelets; translocation of NADase	[13, 14, 115]
Streptolysin S			Damaging of PMNs	Osmotic lysis of erythrocytes; damaging of platelets	[14, 25]
Superantigens (SpeA, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, Ssa, SmeZ)*				Superantigenicity, inducing massive release of inflammatory cytokines	[184]
NAD-glycohydrolase (NADase)				Cytotoxin due to β-NAD cleavage, production of cyclic ADP-ribose, and ADP-ribosyltransferase activity	[187]

*Variable presence among GAS genetic lineages.

Several fibronectin-binding proteins, including some M proteins and SfbI, can also act as invasins [14]. When bound to fibronectin or laminin, GAS invasins interact with host integrins (transmembrane glycoproteins that bind to ECM proteins like fibronectin and laminin), activating a host signal transduction pathway that leads to cytoskeletal rearrangements, resulting in GAS internalization [14, 212]. Although GAS is considered an extracellular pathogen, internalization and intracellular survival of GAS has been shown in several cell types, including keratinocytes and respiratory epithelial cells [103, 133, 148, 153, 212]. Internalization has been proposed as a mechanism to avoid host defense mechanisms, promoting GAS carriage and leading to treatment failures, since the organism would be able to persist protected from phagocytes, humural antibodies, and antibiotics that do not efficiently cross the eukaryotic cell membrane, like penicillin [14]. In favor of this theory, intracellular GAS have been identified in the tonsils of children suffering from recurrent pharyngitis [150]. Internalization has also been proposed as a mechanism to promote the bacterial invasion of deeper tissues [103], which is an important step in the pathogenesis of invasive GAS infections. However, other studies report that strains capable of causing invasive infections are less efficiently internalized by epithelial cells and suggest that cell invasion is a mechanism to promote local persistence of GAS instead of bacterial dissemination [133, 169], so this remains a controversial issue.

The accumulation of plasmin activity on the bacterial cell surface strongly contributes to dissemination of GAS through the tissues, by degrading fibrin clots and ECM components (directly and through the activation of ECM metalloproteases or collagenases). This process is mediated by several plasminogen binding proteins expressed on the surface of GAS cells (Table I-1), and by secreted streptokinase, which activates plasminogen to plasmin [29, 33]. Bacterial spread is also facilitated by the interaction of the hyaluronic acid capsule with the CD44 receptor on keratinocytes, which induces cytoskeletal rearrangements leading to the disruption of intercellular junctions [213], as well as by the activity of secreted enzymes that contribute to the degradation of tissue components and liquefaction of pus, namely DNases, hyaluronidase, and the cysteine protease SpeB [14].

One of the hallmarks of GAS pathogenesis is the ability of this organism to evade the human immune system by several overlapping mechanisms (Table I-1). The hyaluronic acid capsule plays an important role as a physical barrier to complement-mediated phagocytic killing [33]. Moreover, the chemical structure of the capsule polymer is very similar to the hyaluronate of human connective tissue, making it a poor immunogen, thus facilitating GAS evasion of the host immune response [14]. Avoidance of phagocytosis is also achieved

through immunoglobulin cleavage, immunoglobulin binding, direct damaging of phagocytic cells, and through the interaction between several bacterial molecules and host factors leading to the inhibition of the classical and alternative complement pathways and to the blockage of phagocyte recruitment and activation (Table I-1). The M protein is capable of playing multiple roles in this process, as discussed in section 4.1. Extracellular DNase activity, in particular that mediated by the potent DNase Sda1 (also known as SdaD2), has been shown to avoid GAS entrapment in neutrophil extracellular traps (NETs) by degrading the DNA fibers constituting these structures [19]. Another mechanism contributing to enhanced GAS survival and evasion to the host immune system is the degradation or inhibition of host molecules with antimicrobial properties or with chemotactic effects, like the cathelicidin LL-37, lysozyme, defensins, and interferon (IFN)-γ, mediated by the cysteine protease SpeB or the streptococcal inhibitor of complement (Sic), which is present in some GAS lineages [149].

Finally, during the course of infection several GAS components exert a toxic effect in the host (Table I-1). Streptolysins S and O, NAD-glycohydrolase, and phospholipase A2 act directly by damaging epithelial and immune cells [14, 25, 182, 187]. The M protein and the streptococcal superantigens (SAgs) contribute to the induction of a massive inflammatory response, leading to vascular leakage, hypotension, and ultimately shock, multiorgan failure, and death [146, 184]. Spe B contributes to this process through the activation of interleukin (IL)-1β, kinins and histamines, and also through the degradation of fibrinogen, which may interfere with blood clotting [140].

In the next sections, some of the GAS virulence factors are described in more detail due to their relevance in the scope of this thesis.

4.1. M protein

The M protein has long been recognized as a major virulence factor of GAS for its anti-phagocytic properties [101]. It was initially identified due to its type-specific antigenicity, which is the basis for a widely used serotyping method that has been replaced in recent years by the nucleotide-based *emm*-typing scheme (both discussed in section 5) [3, 102].

The M protein is encoded by the *emm* gene, which is part of a family of other *emm*-related genes encoding M-like proteins, called the *emm* gene superfamily. Each strain can have one, two or three *emm* or *emm*-like genes arranged in tandem, near the multiple gene regulator (*mga*), a positive transcriptional regulator that controls the expression of M and M-

like proteins, among other GAS proteins [10, 81]. According to the number and relative chromosomal arrangement of these genes, five major *emm* patterns (termed A-E) have been identified. Isolates of the same *emm* type are nearly always of the same *emm* pattern and there is a strong, though not absolute, association between the *emm* pattern and the primary infection sites of the isolates: strains presenting patterns A, B, and C occur mostly among pharyngeal infections, pattern D isolates are recovered mainly from skin and soft tissue infections, and pattern E strains are called "generalists", since they can be found with similar frequencies in both kinds of tissues [10]. Isolates causing invasive infections usually belong to patterns A-C, suggesting that the nasopharyngeal mucosa is the principal reservoir for invasive GAS strains [59].

In 1986, Hollingshead and colleagues [79] determined the first amino acid sequence of an M protein (M6) and since then the structure of several M proteins has been studied and the sequence of a large number of *emm* genes has been determined [60, 127, 183]. The M protein is composed of two polypeptide chains forming a coiled-coil α-helix that is anchored to the cell membrane and crosses the cell wall, appearing as fibrils on the cell surface. M proteins share a basic structure consisting of a conserved signal peptide, a helical central rod region composed of a variable number of repeat sequences (A, B, C, and D repeats) with a hypervariable N-terminus, and a highly conserved C-terminus (Figure I-2) [60, 183]. The conserved 41-amino acid signal peptide is cleaved during transport to the cell surface and is responsible for targeting the molecule for export and for directing its secretion to the division septum [25, 183]. The hypervariable N-terminal region confers the abovementioned serotype and antigenic specificity. Opsonic antibodies directed against this region override the antiphagocytic mechanisms exhibited by the M protein by activating the classical complement pathway, conferring type-specific immunity [14]. The C-terminal region includes a proline/glycine rich hydrophobic and membrane spanning domain that is anchored to the cell membrane by an LPSTGE motif [60, 146]. Considerable variations in total M protein size, length of the non-helical region at the N-terminus, and size, number, and sequence of A, B and C repeats is observed among distinct M proteins, although there is a strong correlation between these features and the *emm* pattern [127, 183]. Size variation is frequently observed even in M proteins derived from isolates of the same M/*emm* type due to homologous recombination events [60, 127].

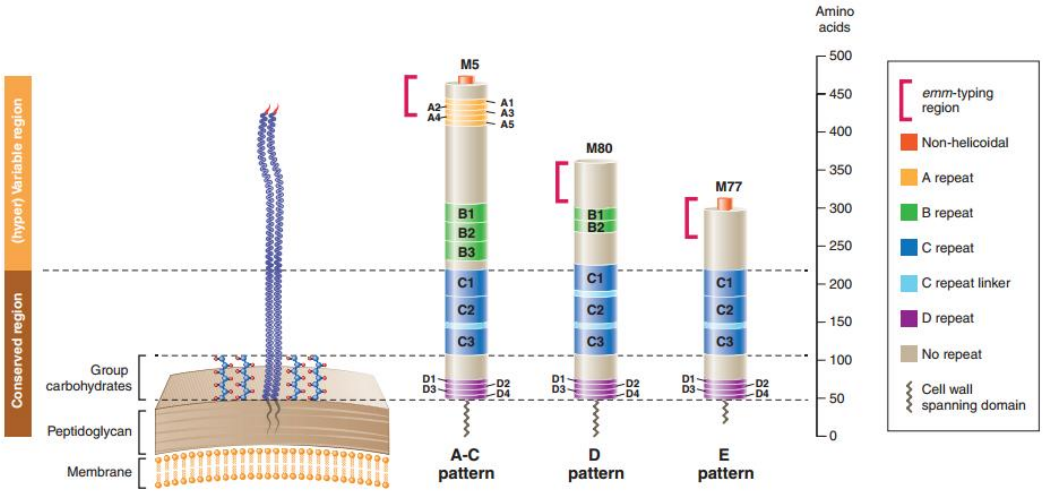

Figure I-2 Representative M protein models. Proteins M5, M80 and M77 were selected as prototypes for the structural characteristics of each *emm* pattern group. M protein length and the size of the repeat and non-repeat regions are drawn to scale. Reproduced from [127].

As shown in Table I-1, M protein plays multiple roles in the pathogenic mechanism of *S. pyogenes*. Notably, not all M proteins are capable of the same functions, translating the structural differences observed between distinct M proteins, possibly associated with different host-imposed selective pressures and adaptation to specific tissues. Different M proteins are capable of binding a large number of host proteins, including fibronectin, kininogen, fibrinogen, immunoglobulins, albumin, complement-binding proteins, and cell-surface receptors like CD46 [146, 183]. M protein functions may be further influenced by the protein folding state, which modulates the binding affinity to host factors. Protein folding has been shown to be temperature-sensitive and may also be affected by interactions with host proteins [183], so that the same M protein may perform different functions according to the host tissue where the bacteria are found.

The M protein has been suggested to promote GAS adherence to host cells by binding fibronectin (fibronectin-binding has been shown for M1, M3, and M6 proteins), the CD46 receptor on keratinocytes, and glycosaminoglycans on epithelial cells and skin fibroblasts. It may also play a role in GAS internalization, since complexes of M protein with fibronectin or laminin interact with integrins on human epithelial cells, leading to internalization of GAS [146]. M proteins belonging to pattern D contribute to bacterial dissemination through the tissues by binding plasminogen and participating in the accumulation of cell-surface plasmin activity, which degrades fibrin clots and ECM components [183]. Concerning immune evasion, M proteins play a key role in protecting *S. pyogenes* from phagocytosis, participating in several mechanisms to inhibit both the classical and the alternate complement pathways

(Table I-1), while reducing the binding of serotype-specific antibodies. Moreover, some M proteins, especially those belonging to pattern E, and some M-like proteins bind to the Fc region of immunoglobulins, potentially contributing to phagocytosis inhibition [146, 183]. Most M proteins bind kininogen, possibly leading to bradykinin release at the bacterial surface, contributing to vasodilatation and vascular leakage [183]. Several other pro-inflammatory properties have been attributed in particular to the M1 protein (Table I-1), which may partly explain the association of M1 isolates with the occurrence of STSS (section 6) [146, 183]. The superantigenic activity of the M proteins is still controversial and may be influenced by their three-dimensional structure [146], being hard to predict from the primary sequence.

As previously mentioned, molecular mimicry of M proteins is involved in acute glomerulonephritis and, especially, acute rheumatic fever. Antistreptococcal antibodies cross-react with several human α-helical coiled-coil proteins like cardiac myosin, tropomyosin, vimentin, laminin, keratin, several valvular proteins, and proteins from the glomerular basement membrane. Moreover, direct binding of collagen to M and M-like proteins has also been implicated in the induction of acute rheumatic fever [146].

4.2. Pili

In 2005, Mora and collaborators [135] reported the identification of pilus-like structures on the surface of GAS strains, encoded in a chromosomal island previously described as FCT region (Fibronectin-binding, Collagen-binding, T antigen) [11]. Nine different FCT types have been identified, with a varying presence and organization of genes encoding the pilus proteins, ECM-binding proteins, transcriptional regulators, sortases, signal peptidases, putative proteins of unclear function, and mobile genetic elements (MGEs) [96, 195]. GAS pili are composed of a backbone protein and one or two ancillary proteins. The backbone protein was identified as the trypsin-resistant T antigen that has long been used for serotyping purposes, as discussed in section 5.1 [135].

The presence or absence of certain genes within the FCT region, in particular those encoding fibronectin- and collagen-binding proteins, have been shown to significantly correlate with certain *emm* patterns, suggesting that the FCT region, which is highly prone to genetic recombination events, may be involved in GAS adaptation to specific host tissues [95].

Pili have been suggested to promote GAS colonization, since they play a role in bacterial attachment to pharyngeal epithelial cells and keratinocytes [1, 2, 119], and are involved in self-aggregation, formation of microcolonies, and biofilm production [118, 119]. However, their role in virulence remains unclear. At least in the M1T1 background, pili have been shown to stimulate IL-8 induction and to promote the entrapment of the pathogen in NETs, reducing virulence in murine models of infection [2]. Moreover, the role of pili in attachment and colonization appears to be variable. Experiments with an M49 GAS isolate showed no influence of pili in attachment and internalization in epithelial cells, production of biofilms, nor survival in human blood, and pili expression in this isolate was also associated with attenuated virulence in a murine model of skin infection [139]. Thus, the role of pili in adhesion and virulence is probably serotype- or even strain-specific, depending on the composition of the FCT region and possibly on a differential regulation of genetic expression throughout the infectious process.

4.3. Streptococcal superantigens (SAgs)

The streptococcal SAgs are ~22-28 kDa proteins with signal peptides that are cleaved upon secretion [125]. They share amino acid sequence identities between 17% and 48%, and are part of a larger family of toxins that includes the structurally related staphylococcal SAgs [161]. Streptococcal pyrogenic exotoxins (Spes) A, B, and C, also known as erythrogenic toxins, were initially identified due to their ability to induce fever and to their association with the erythematous rash characteristic of scarlet fever [161, 184]. In 1990, the superantigenic role of streptococcal pyrogenic toxin A (SpeA) was demonstrated for the first time [84].

SAgs are extremely potent inflammatory inducers, due to their ability to interact with the major histocompatibility complex (MHC) class II molecules outside the peptide-binding groove and with the variable region of the T cell receptor (TCR) β-chain, without previous processing by antigen-presenting cells, in contrast to conventional antigens (Figure I-3). Each SAg is therefore able to activate a large number of T cells expressing a characteristic Vβ signature, regardless of the antigenic specificity of their TCR, leading to a massive secretion of proinflammatory cytokines (IL-1, IL-6, tumor necrosis factor (TNF), IFN-γ) [93, 184]. The release of excessive amounts of these immunomodulators may activate the complement, coagulation, and fibrinolytic cascades, causing the hypotension and multiorgan failure observed in STSS [14].

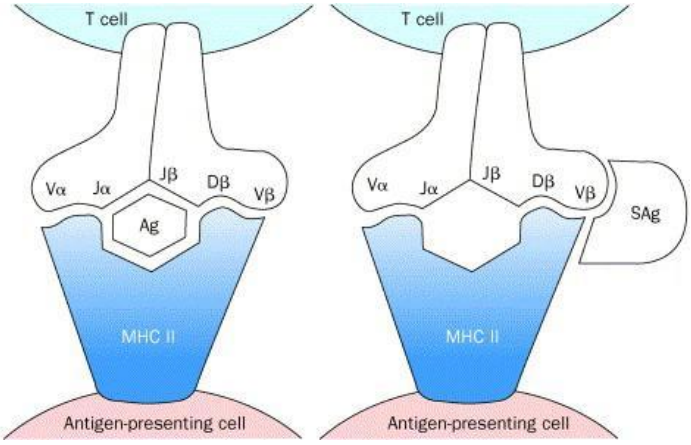

Figure I-3 Interaction of a conventional antigen (Ag, left) and a superantigen (SAg, right) with the MHC class II and the T cell. The SAg does not require processing by the antigen-presenting cell, binding the MCH class II outside the peptide binding groove, and interacting with the Vβ region of the TCR. Adapted from [14].

A total of 13 pyrogenic toxins have been described in GAS to date: SpeA, SpeB, SpeC, SpeF, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SSA (streptococcal superantigen) and SMEZ (streptococcal mitogenic exotoxin Z). Most of the genes encoding these toxins are located on prophages, namely *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, and *ssa*, while others (*speB*, *speF*, *speG*, *speJ*, and *smeZ*) are generally considered to belong to the bacterial core genome, since they are not found in association with bacteriophage genes [125]. SpeB and SpeF have been shown to correspond to a cysteine protease and a DNase, respectively, and the mitogenic activity initially described for these toxins was later shown to be due to contamination of the protein extracts with other SAgs [18, 67, 90].

Several epidemiological studies indicate that the production of superantigenic toxins by GAS is associated with scarlet fever, STSS, and post-infection sequelae [41, 74, 108, 137, 216]. Animal experiments have yielded contradictory results, with some studies confirming a role of SpeA in the development of STSS and others finding no influence of this toxin in infection, but these discrepancies can be caused by the different animal and infection models used [25, 161]. However, clinical studies involving the detection of SAgs, mitogenic activity, or SAg-neutralizing antibodies in patient sera support an association between GAS SAgs and severe invasive infections, in particular for SpeA and SmeZ [161].

The different SAgs produced by GAS have different Vβ specificities, thus inducing different T-cell subsets [161]. Moreover, the genes *speA*, *speC*, *speG*, *ssa*, and *smeZ* have been shown to present multiple allelic variants that may be associated with differences in their superantigenic activity, as well as in antigenic properties [9, 184]. Of all the studied SAgs, SMEZ is the most potent in inducing cytokine production *in vitro* and has also been shown to be a major trigger of the inflammatory response during experimental streptococcal sepsis

[184]. This SAg seems to be the main contributor to the mitogenic activity of sera collected from patients with STSS, and it has been recently reported that *emm*3 isolates show a considerably reduced mitogenic activity than other isolates causing STSS due to a naturally occurring mutation that inactivates *smeZ* [162, 199]. This gene is highly polymorphic, with 50 allelic variants available in GenBank to date, presenting significant mosaic structure, which indicates that *smeZ* variants arise mostly by recombination events [161]. The development of neutralizing antibodies against SMEZ after GAS infection has been reported and the lack of antibodies against SAgs has been suggested as a risk factor for the development of invasive GAS disease [161, 162].

Different SAgs bind more avidly to B cells expressing particular human leukocyte antigen (HLA) class II alleles [110, 142]. In agreement, some HLA class II haplotypes seem to confer a greater predisposition for the development of serious invasive GAS infections, while others confer protection, and others are apparently neutral [94]. Therefore, the magnitude of the inflammatory response to a GAS infection is probably influenced by the host HLA haplotype, the repertoire of SAgs expressed by the infecting strain, and the presence of antibodies against these SAgs.

4.4. Transcriptional regulation of virulence factors

S. pyogenes presents a complex network of transcriptional regulators, including stand-alone regulators and two-component systems (TCSs), which interact with each other in order to promote a coordinated differential expression of virulence genes important for the distinct stages of the pathogenic process, and of genes involved in metabolism and resistance to environmental stress. This intricate regulatory network may allow the pathogen to rapidly respond to environmental changes and adapt to specific host niches and infection types. The regulatory mechanisms of GAS are highly variable between strains of different genetic lineages, and are also considerably affected by the environment, the growth conditions, and the stage of growth of the bacterium [126, 138].

More than 100 putative stand-alone regulators have been identified, of which three have been extensively studied, namely the multiple gene regulator Mga, Rgg (also known as RopB), and the RofA-like protein family of regulators (RALPs), which includes RofA, Nra, Ralp3, and RivR [126, 138]. Stand-alone regulators are closely linked to GAS metabolism in order to respond to growth changes [126]. A model of growth-phase-dependent activation of

stand-alone regulators has been proposed, in which Mga is active during the exponential growth phase, promoting the transcription of genes involved in GAS colonization, RALPs are expressed in the transition to the stationary phase, shutting down Mga and changing the transcriptome in a way that favors GAS persistence, and Rgg is active during the stationary phase, keeping Mga downregulated and promoting GAS spread. This regulatory baseline may be further adjusted and modified by TCSs, which are able to sense extracellular signals, allowing the pathogen to alter its transcriptome in response to environmental changes or interactions with the host [97].

A TCS is typically composed of a transmembrane protein that acts as a sensor histidine kinase and a cytoplasmic DNA-binding response regulator. Upon sensing a specific extracellular signal, the kinase alters the phosphorilation state of the response regulator, leading to alterations in gene expression in response to environmental signals [89]. Based on the published complete GAS genomes, 13 conserved TCSs have been identified [138]. Most of them have now been studied with some detail, although three have been known for a longer time and have been studied more thoroughly, namely CovR/S (also known as CsrR/S), Ihk-Irr, and FasBCA [97, 138]. Current knowledge indicates that these regulatory systems are crucial for the coordinated expression of genes encoding virulence factors, in response to several environmental stimuli and at least some of them regulate or interact with other transcriptional regulators, both stand-alone and TCSs [69, 138]. The CovR/S system has been intimately associated with the development of invasive GAS infections. This TCR has been shown to directly or indirectly regulate approximately 15% of the GAS genome, including genes that encode important virulence factors, like the hyaluronic acid capsule, SpeB, streptokinase, streptolysins, streptodornases (including Sda1), NADase, SpyCEP, Mac, and C5a peptidase [53, 69, 191, 197]. The acquisition of spontaneous mutations in the *covS* or *covR* genes, resulting in the up-regulation of most of the abovementioned virulence genes, but in the downregulation of *speB*, has been associated with a shift from a non-invasive to an invasive phenotype [191]. In agreement, mutations in these genes and in the Rgg stand-alone regulator have been found with a higher prevalence among isolates associated with STSS than in isolates recovered from non-invasive infections [83].

5. Epidemiological typing of GAS

Typing of bacterial strains is widely used in surveillance epidemiology as a means of differentiating related bacterial isolates, with the aim of recognizing outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of infection, controlling vaccination programs, and monitoring the geographical and temporal dissemination of clones of isolates with specific properties, like antibiotic resistance or increased virulence [147]. Moreover, bacterial typing is an important tool for the studies regarding bacterial population structure, dynamics and evolution, and, in association with clinical epidemiological data, can contribute to investigations on the pathogenesis of infectious disease [204]. This typing can be achieved by phenotypic methods, relying on expressed bacterial features (for example, antibiotic resistance, antigenic properties, susceptibility to bacteriophages, production of bacteriocins), or by genotypic methods, discriminating the isolates based on the genomic differences between them. Phenotypic methods are useful for a rapid identification of outbreak situations in clinical laboratories because they are usually simpler and less expensive. However, they are being largely replaced or complemented by genotypic methods, since most of the phenotypic methods are not suited for evolutionary studies and present serious limitations for infection control and surveillance purposes, due to insufficient discriminatory power, to reproducibility issues caused by a variable phenotype expression, and to a rapid alteration of some phenotypic properties caused by selective pressure [54, 204].

5.1. Serotyping

Serotyping of GAS based on agglutination of the surface antigen T (T typing) and on precipitation of the M protein (M typing) has long been used in the epidemiological surveillance of GAS infections [44]. T typing is based on the resistance of the T antigen to trypsin digestion. Most GAS strains have a well defined T type antigen and usually a limited number of T types are associated with each M type [88]. However, this method presents reproducibility issues, since it is affected by several factors, including the growth temperature, the source of typing sera (different manufacturers have slightly different serotyping schemes), and the extent of trypsinization [88, 151]. Moreover, some studies report a considerable proportion of non-typeable isolates [48, 88, 112]. The interest in T-typing has been renewed

since the identification of the T antigen as the main structural protein of GAS pili [135], and a molecular, polymerase chain reaction (PCR)-based method for T-typing has been proposed [52].

M typing was developed decades ago by Lancefield [102] and was extensively used, but this method presents practical disadvantages, given the difficult preparation and maintenance of the sera and the growing number of M types identified (currently more than 80 unique M types), each requiring a specific serum. Moreover, there is a high level of non-typeable isolates due either to the lack of specific sera or to the limited expression of the M protein [3, 51]. Serum opacity factor (SOF) typing can be used in association with M typing to overcome some of the technical difficulties of the latter method. SOF is a lipoproteinase that increases the opacity of mammalian serum and that is produced by approximately 50% of GAS strains. Antibodies against the SOF are type specific and correlate with the M type, usually allowing the identification of the M type by determining the SOF type in SOF-positive isolates [33]. However, exceptions have been reported, in which isolates of different M types share the same SOF type [4].

5.2. *emm* typing

Given the limitations associated with M serotyping, this method has been largely replaced by the analysis of the *emm* gene region encoding the N-terminus determining the serospecificity of the M protein [3]. The method consists in the amplification of a large portion of the *emm* gene by PCR, followed by direct sequencing of the 5' hypervariable region. The resulting sequence is searched against a type-specific DNA sequence database using a specific BLAST tool available at the internet site of the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm), and the strain is assigned an *emm* type number. For the typing of large numbers of isolates, amplicon restriction profiling can be used in association with other methods, like T-typing, to assign the *emm* type, avoiding the sequencing of the *emm* gene for all isolates (http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm). According to the CDC database, there are currently more than 200 different *emm* types associated with GAS. Many of them have a direct correspondence with an M serotype, but newer *emm* types (> *emm*93) were identified in isolates non-typeable with any of the M typing sera available in international GAS reference laboratories. On the other hand, some strains that were known to

serotype with distinct M-type sera were found to have an identical N-terminal *emm* sequence, now being classified in the same *emm* type. This is the case of M44 and M61 isolates, which were identified as *emm*44/61 and are now designated simply by *emm*44 (http://www.cdc.gov/ncidod/biotech/strep/types_emm103-124.htm).

Although *emm* typing became the gold standard for comparing and differentiating GAS strains isolated in epidemiological studies, it has been shown that it should be complemented with other typing methods, in order to unambiguously identify GAS clones [22].

5.3. Pulsed field gel electrophoresis (PFGE) macrorestriction profiling

PFGE was widely adopted as the primary typing method for the epidemiological surveillance of bacterial infections during the 1990s and remains the most frequently used tool for the characterization of bacterial isolates in outbreak contexts [68, 167]. The success of PFGE as a typing method is due to its excellent discriminatory power, high epidemiological concordance, excellent typeability, high intra-laboratory reproducibility, and relatively inexpensive protocol, although it is technically demanding, labour-intensive, time consuming, and the resulting data present a limited portability [167].

The method consists on digesting total bacterial DNA embedded in agarose plugs with an endonuclease with an infrequent recognition sequence, and separating the resulting fragments in an agarose gel subjected to electric pulses of varying directions [68]. In most cases, this approach addresses >90% of the bacterial genome and allows the detection of insertions or deletions of MGEs and large recombination events [167]. For *S. pyogenes*, the most commonly used endonuclease is SmaI, although the DNA of GAS isolates expressing the M phenotype of macrolide resistance (section 7.1.1) is usually not digested with this endonuclease due to the presence of a methyltransferase in the same genetic element containing the *mef* gene [58]. This problem can be solved by the use of Cfr9I, an isoschizomer of SmaI that is insensitive to the DNA modification introduced by the methyltransferase [179].

The genetic relatedness of the isolates is determined based on the comparison of the band profiles obtained for each isolate (Figure I-4), since the number of genetic events separating two isolates can be estimated from the number of band differences between the two corresponding profiles [196]. Large numbers of isolates can be easily compared using

informatic tools that automatically assign bands and determine strain relatedness using algorithms to produce a numerical representation of PFGE banding pattern similarity [68]. Most commonly, the unweighted pair group method with arithmetic means (UPGMA) is used to create dendrograms based on Dice similarity coefficient values, and an 80% cutoff value has been shown to be appropriate to define clusters of related isolates when analyzing SmaI Dice/UPGMA dendrograms of GAS isolates [22].

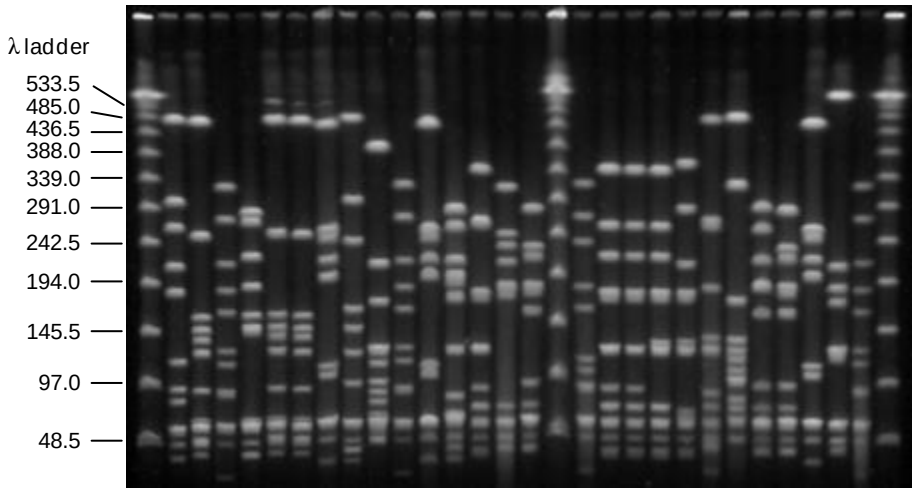

Figure I-4 Pulsed-field gel electrophoresis (PFGE) profiles generated following SmaI/Cfr9I digestion of total DNA from *S. pyogenes*. Fragment sizes (kb) of the Lambda (λ) PFGE marker (New England Biolabs, Beverly, USA) are presented.

SmaI/Cfr9I PFGE profiling provides a more accurate characterization of GAS clones than *emm* typing alone, and the *emm* type of a given isolate can be confidently predicted from its PFGE profile, while the converse relationship is rather weaker [22].

5.4. Multilocus sequence typing (MLST)

MLST is a sequence-based typing method that is widely used for a large number of bacterial species [167]. Typically, it involves the direct sequencing of internal fragments of seven housekeeping genes, which are previously amplified by PCR, and matching the results with a central and publicly available database (in the case of *S. pyogenes*, http://spyogenes.mlst.net/). Each of the seven alleles is assigned a number, defining the strain's allelic profile, which in turn is assigned a sequence type (ST) number. This provides highly reproducible, unambiguous, and portable data, facilitating the comparison with other studies and the evaluation of the geographical dissemination of the identified clones [47]. The

use of slowly evolving genes renders this method suited for the reconstruction of evolutionary events, at least within short time scales, although it may fail to detect the variability of closely related strains that may be required for outbreak studies [54, 204].

The eBURST algorithm is frequently used to define clonal complexes of closely related isolates, based on their allelic profiles, and graphically represent the genetic relatedness between these isolates [55]. A clonal complex is defined as a group of isolates that share 100% identity at six or seven MLST loci with at least one other member of that group. The founding genotype is assumed to be the one sharing six of the seven alleles with the largest number of isolates belonging to the same clonal complex [54]. According to this model, a founding genotype can originate a clonal complex by diversification while increasing its frequency in the population, due either to a fitness advantage or to random genetic drift [54, 55]. Diversification of the founder genotype initially occurs due to changes in one of the alleles (by point mutation or recombination), originating single-locus variants (SLVs). Eventually, SLVs diversify further to produce double-locus variants (DLVs), triple-locus variants (TLVs), and so on. For each clonal complex, eBURST determines a hypothetical pattern of descent, displaying links between the STs [55]. However, the eBURST is a locally optimized algorithm, which can result in links that violate the rules proposed. A solution to this problem has been suggested, consisting on a globally optimized implementation of the eBURST algorithm (goeBURST), which guarantees a unique solution for the BURST rules, with the advantage of allowing a visual evaluation of the reliability of the represented hypothetical pattern of descent [63]. The resulting data can be dynamically integrated with other epidemiological or molecular typing data (like antimicrobial resistance, isolation source, or *emm* type, for example), using the PHYLOViZ software [64].

The MLST ST has been shown to be a very good predictor of the *emm* type, but the converse is not always observed, since many *emm* types occur in more than one ST or even in more than one clonal complex, probably due to the horizontal transfer of *emm* genes between distinct genetic lineages [12, 22]. Therefore, MLST analysis allows the differentiation of isolates belonging to the same *emm* type and provides a more consistent clone definition. In spite of the determinant role of the horizontal exchange of large genomic fragments for the diversification of GAS (section 8), MLST has been reported to generate simillar results to SmaI/Cfr9I PFGE profiling when defining GAS lineages [22].

5.5. Superantigen gene profiling

The determination of the SAg gene content of GAS isolates is frequently used as a complementary typing method [30, 38, 108, 120, 131]. The screening of the SAg genes is usually performed by either single or multiplex PCR reactions [108, 131], and the resulting pattern of present and absent genes defines a SAg gene profile. The interpretation of multiplex PCR results can be complex due to the high number of simultaneously amplified DNA fragments that need to be differentiated. However, multiplex reactions have the advantages of reducing the time and cost of the method and allowing the simultaneous amplification of a DNA fragment known to be present in all GAS strains, which is used as a control of DNA quality. The primers used for these reactions must be carefully designed, so that the resulting fragments can be separated by agarose gel electrophoresis and all known allelic variants of the genes can be amplified, avoiding the occurrence of false negatives. On the other hand, primers that may amplify non-functional SAg gene remnants [34] or that may unspecifically anneal with other genome regions must be avoided, in order to prevent the occurrence of false positives. The use of diverse primers, some of which not ideally suited for the PCR-screening of all described SAg gene variants, together with a confusing gene nomenclature [161], has confounded the comparison of SAg profiling results from different studies.

Not surprisingly, the phage-encoded SAg genes are found with a highly variable frequency in different GAS lineages, while the genes *speG* and *smeZ*, which are encoded on the bacterial core genome, are present in the great majority of the isolates [30, 131]. The *speJ* gene is also generally considered to belong to the bacterial core genome, but while this gene has been identified in all analysed isolates of a collection comprising diverse *emm* types in at least one study [45], several other works reported its absence in a large number of strains [30, 128, 131]. Therefore, the origin of the *speJ* gene remains controversial.

The rationale for using SAg gene profiling in GAS typing is linked not only with their role in virulence and potential association with STSS and scarlet fever [41, 74, 108, 137, 216], but mostly with the fact that the phage-encoded SAgs can be considered as markers for the presence and transfer of prophages that can also carry other genes conferring specific phenotypic properties to GAS clones. In fact, lateral gene transfer events involving bacteriophages and other MGEs have been identified as the major mechanism driving the genomic diversification of GAS (section 8) [7, 128, 190, 209]. The usefulness of the SAg gene profiling as a typing method for GAS has not been comprehensively evaluated and remains questionable, since some studies emphasize the strong association between the SAg

profile and the *emm* type [164, 168, 208], while others show that SAg gene profiling can help to further discriminate GAS isolates analyzed by *emm* typing [38, 108, 120, 131]. Likewise, conflicting results regarding the association between specific SAg genes and invasiveness have been reported [31, 41, 108, 120, 166, 168]

5.6. Other typing methods

In the previous sections, the typing methods most commonly used for the characterization of GAS isolates were described. However, many other molecular methods have been used to some extent in GAS epidemiological typing. These include: *vir* typing, a restriction fragment length polymorphism (RFLP) analysis of the *vir* (*mga*) regulon of GAS [66]; random amplification of polymorphic DNA (RAPD) [170]; restriction endonuclease analysis (REA) of genomic DNA [171]; RFLP analysis of rRNA genes (ribotyping) [171]; fluorescent amplified fragment length polymorphisms (FAFLP) analysis [40]; multilocus enzyme electrophoresis (MLEE) [137]; genotyping of the *sic* gene in *emm*1 isolates [76]; sequence-based typing of the SclB gene, which encodes a collagen-like protein [27]; multilocus variable number tandem repeat analysis (MLVA) [145]; phage profiling [17].

In recent years, the development of faster and cost-effective next generation sequencing methods has provided means for obtaining nearly complete genome sequences of increasing numbers of bacterial strains. Whole-genome sequencing (WGS) has been successfully used for both long-term epidemiological studies and for clarifying outbreak situations associated with several different bacterial pathogens [23]. Beres and colleagues used genome-wide single-nucleotide polymorphism (SNP) comparisons to understand the molecular complexity underlying the occurrence of three successive epidemics of invasive GAS infections caused by *emm*3 isolates in Canada [6], as well as to track the rapid emergence and spread of a highly virulent *emm*59 clone [61, 62]. WGS has also been used to compare GAS isolates associated with a puerperal sepsis cluster occurring in a six month period in Sydney [5], and to trace the evolutionary events leading to the emergence of the highly successful M1T1 clone [114].

5.7. Evaluation and comparison of typing methods

The appropriateness of a method for typing a specific organism with a given purpose should be evaluated and validated according to a number of proposed criteria [204]. One of the most important aspects in the context of both epidemiological and evolutionary studies is the discriminatory power of the method, which refers to the ability of assigning to different types two strains randomly sampled from the population. The discriminatory power can be measured by the Simpson's index of diversity (SID) and respective confidence interval, with higher values (>0.95) indicating a good discriminatory power [22, 204]. This index can also be used to evaluate the diversity of a population of isolates regarding a given property, characterized by a specific typing method.

Epidemiological concordance is another important aspect to consider, since the results of a typing method should reflect the available epidemiological information of the isolates [204]. Concordance between distinct typing methods can be evaluated using the Wallace (W), and adjusted Wallace (AW) coefficients [22, 172], which provide an estimate of, given a typing method, how much new information is obtained from another typing method [22].

The SID, Wallace, adjusted Wallace, and their respective 95% confidence intervals, among other measures, can be easily calculated for any given dataset using an online tool available at http://www.comparingpartitions.info/.

6. Epidemiology of invasive GAS infections

The global burden of invasive disease caused by GAS is very hard to assess due to the lack of consistent and accurate data. Most countries do not have a surveillance system implemented and the majority of the published studies rely on voluntary reporting systems [21, 98]. The lack of information is particularly evident in developing countries, where the infections caused by this pathogen are estimated to have a higher prevalence [21]. An additional problem is the definition of invasive infection, which differs among distinct studies. Some consider as invasive only the infections in which GAS is isolated from a normally sterile site, while others include other isolation sources when accompanied by systemic signs of invasive infection [98].

In industrialized countries, the incidence and severity of GAS infections decreased for most of the 20th century, presumably due to antimicrobial therapy use and to a general

improvement in socio-economic and sanitary conditions. However, a reemergence of GAS invasive disease has been observed since the late 1980s, both in North America and in Europe [33, 44]. Between 1990 and the early 2000s, the estimates for the incidence of invasive GAS disease varied considerably among different European countries, although most of them reported rates of 2.7-3.6/100 000 inhabitants per year. Remarkably, in most of the countries with published results for at least five consecutive years, there was a global increasing trend in the incidence of invasive GAS infections throughout the 1990s [98]. In 2003 and 2004, the Strep-EURO program attempted to perform a standardized surveillance of invasive GAS disease involving 11 European countries. Reported incidence rates varied between 0.3/100 000 in Cyprus and 2.94/100 000 in the United Kingdom. Marked differences were observed between the incidence rates registered in Northern European countries (2.19-2.94/100 000) and all the remaining countries (0.3-1.48/100 000), which are probably related with a higher compliance with the reporting system and with a more frequent use of microbiological investigative methods in the former countries [99]. Recent studies coming mostly from Northern Europe, France, and Spain indicate that during the decade of 2000 the incidence of invasive GAS disease remained high in Europe [38, 91, 107, 113, 131, 134, 176]. In the U.S.A., a stable average annual incidence of 3.5/100 000 was reported between 1995 and 2005 [143, 144], and in Canada studies from different regions reported rates between 2.4 and 4.8/100 000 in time periods comprised between 1995 and 2004 [80, 104, 201]. In 2005, based on a compilation of the available reports of invasive GAS disease from different countries throughout the world, Carapetis and colleagues [21] estimated an overall GAS invasive disease incidence of 2.45/100 000 for the developed countries, with a median case fatality rate of 15%. However, as mentioned in section 3.2, mortality can reach much higher values when associated with certain clinical manifestations, like pneumonia, necrotizing fasciitis, and especially STSS and myositis [189].

Several studies suggest a correlation between invasive infection and specific epidemiological markers. In most countries, M/*emm* types 1, 3, and 28 have been traditionally associated with invasive GAS disease [46, 80, 143, 207]. In addition, associations between certain M/*emm* types and specific invasive infections have also been reported, namely between M/*emm*28 and puerperal infections, M/*emm*1 and STSS, and M/*emm*3 and necrotizing fasciitis [143, 201, 207]. The clone characterized by *emm*1-T1-ST28, commonly designated by M1T1 clone, has remained the dominant lineage associated with invasive GAS infections in North America and Europe since the 1990s, with average prevalence rates of 22% and 19%, respectively, according to the most recent estimates [112, 144]. However,

recent studies indicate some temporal changes in the clonal composition of the invasive isolates in these regions. In Northern European countries, namely Finland, Denmark, Sweden, and Norway, *emm*28 has been the most prevalent type, although its frequency decreased during recent years in Finland [112, 131, 176]. This decrease was accompanied by a rise in the prevalence of *emm*1, which became the most common *emm* type in 2007, and of *emm*84, which emerged in Finland only in 2004, but in 2007 was already the second leading *emm* type [176]. A contrasting situation was reported in Germany and among adults in France, where in recent years *emm*1 decreased, while *emm*28 increased [85, 160]. In Northern and Western Spain, between 1998 and 2009, *emm*1 presented an increasing trend, in contrast with *emm*28 that decreased. In addition, the authors reported the emergence of an *emm*89-ST101 clone in 2002-2005, which increased slightly in 2006-2009 [134]. This *emm* type was the most prevalent among invasive GAS isolates recovered in Sweden between 2002 and 2004 (15.7%) [38], and also increased in the U.S.A. from 1995-1999 to 2000-2005 [143, 144], while in Italy there was a pronounced decrease in the prevalence of a macrolide-resistant clone of *emm*89 isolates from 1994-1996 to 2003-2005 [32]. In recent years, *emm*59, previously rare in developed countries, emerged rapidly in Canada causing an epidemic of severe invasive GAS infections [202]. This clone disseminated also to the U.S.A., where it appears to be genetically diversifying [62].

Generally, in tropical and developing regions the clonal composition of the isolates responsible for invasive GAS infections is markedly different from the one observed in industrialized, temperate countries, presenting a higher diversity of *emm* types and a dominance of types that are not commonly isolated in developed countries [186].

The association between the increase in the incidence of invasive GAS infections and specific GAS clones in developed countries may be due to a higher prevalence of these clones in the general GAS population or to the emergence of lineages with enhanced virulence that become more successful in causing invasive disease. This question has been addressed by many studies attempting to compare the characteristics of the isolates causing invasive and non-invasive infections in the same time period and geographic region, most of which were limited in the number and diversity of isolates analyzed or in the typing methodologies used [31, 41, 45, 48, 108, 111, 123, 164, 166, 200, 210]. Still, although some of them could not find significant differences between the main lineages causing invasive and non-invasive infections [41, 123, 164, 166], others reported relevant differences in the distribution of M/*emm* types, T types, or SAg genes. Isolates presenting *emm*1-T1 have been found with a higher prevalence among invasive infections in Denmark, France, Sweden, Italy, and Japan

[31, 45, 48, 111, 210]. Strains belonging to *emm*28-T28 have been significantly associated with invasive infections in Sweden, while in France these characteristics were reported with a higher frequency among non-invasive isolates [48, 111]. There are also reports of significant associations between the presence of specific SAg genes and invasive GAS disease, especially for *speA* [31, 45, 108, 200]. In contrast, characteristics like *emm*4, *emm*77, and the presence of *speC*, *speH*, or *ssa* have been significantly associated with isolates causing non-invasive infections [31, 45, 108].

7. Treatment, prevention and control of GAS infections

7.1. Antimicrobial therapy

Antibiotic therapy is the first-line treatment for GAS disease, except for deep soft-tissue infections, like necrotizing fasciitis, which require surgical debridement as the major therapeutic approach, although also combined with antimicrobial administration [188]. Despite being a self-limited infection in imunocompetent individuals, the use of antimicrobials in streptococcal tonsillo-pharyngitis cases is important, not only to speed up the relief of symptoms, but especially to prevent non-suppurative complications, in particular rheumatic fever [175]. Moreover, patients with a history of rheumatic fever are indicated for long-term antibiotic prophylaxis in order to prevent recurrence of the disease [24].

Streptococcus pyogenes is uniformly susceptible to penicillin, which is therefore the antimicrobial of choice for the treatment of most uncomplicated streptococcal infections [175, 188]. However, in the treatment of severe GAS infections, like necrotizing fasciitis and STSS, the efficacy of penicillin alone has been questioned, and the association of penicillin with clindamycin is recommended [188]. The higher efficacy obtained when using clindamycin is thought to be associated with a capacity of this drug to suppress toxin release and to modulate cytokine production [188]. Clindamycin and macrolides (clarithromycin or azithromycin) can also be used as alternative choices for penicillin-allergic patients, although resistance to these antimicrobials has been described in several countries [163, 179] (section 7.1.1). Other possible alternatives for these patients are first generation cephalosporins in cases of pharyngitis (for those not anaphylactically sensitive), and vancomycin, linezolid, daptomycin, or quinupristin/dalfopristin for severe soft tissue infections [175, 188].

7.1.1. Antimicrobial resistance

In *S. pyogenes*, resistance to clindamycin and macrolides is particularly important due to the common use of these antimicrobials as therapeutic alternatives for penicillin-allergic patients and for the combined treatment of severe infections, as explained above. Two major macrolide resistance mechanisms are recognized in GAS, resulting in different resistant phenotypes. One is the post-transcriptional methylation of the ribosomal target of these antibiotics (23S rRNA), resulting in a cross-resistance to all macrolides, lincosamides (e.g., clindamycin, lincomycin), and streptogramins B (e.g., quinupristin) – MLS_B phenotype. This phenotype can be expressed either constitutively ($cMLS_B$) or inducibly ($iMLS_B$). In the inducible resistance, the native mRNA produced by the bacteria is unable to encode a methylase, but becomes activated in the presence of a macrolide inducer [105]. The ribosomal methylases are encoded by the *erm* genes (erythromycin ribosome methylase). More than 20 classes of *erm* genes have been identified, but in *S. pyogenes* the MLS_B phenotype is typically associated with either *erm*(B) or *erm*(TR), which is a subclass of the *erm*(A) class [105, 205]. The *erm*(B) genes are usually associated with high-level resistance and can be expressed constitutively or inducibly, while *erm*(TR) is generally inducible and the resulting level of resistance is thought to depend on the contribution of a drug efflux pump [205].

Active antibiotic efflux is the other macrolide resistance mechanism common in streptococci, mediated by efflux pumps that confer low to moderate levels of resistance to 14- and 15-membered lactone ring macrolides (e.g., erythromycin, clarithromycin, azithromycin), but not to lincosamides, nor streptogramins B – M phenotype [105]. In *S. pyogenes*, these pumps are generally encoded by the *mef*(A) gene, although other *mef* variants have been recognized in some strains [39, 205]. GAS isolates harboring a combination of *erm* and *mef* genes have been identified, presenting an MLS_B phenotype [105].

The *erm* and *mef* genes of streptococci are encoded on MGEs, which favors the lateral transfer of resistance, both intra- and inter-species. Moreover, these elements often carry genes conferring resistance to other antimicrobials, frequently tetracycline [205]. The *tet*(M) and *tet*(O) genes, which can both be found in association with macrolide resistance genes in transposable elements in *S. pyogenes*, encode ribosomal protection proteins that allosterically prevent the binding of tetracycline molecules to the ribosome, without interfering in protein synthesis. This is the most common mechanism of tetracycline resistance in *Streptococcus*, although efflux pumps encoded most commonly by the *tet*(K) and *tet*(L) genes can also be found [165]. Due to the common linkage between macrolide and tetracycline resistance

determinants, studies aiming at evaluating the macrolide resistance of GAS isolates often test for tetracycline resistance as well, even though tetracycline is not a therapeutic option for the treatment of GAS infections.

Macrolide resistance rates among GAS isolates vary considerably among different countries [163]. In Portugal, the erythromycin resistance rate among *S. pyogenes* isolates associated with tonsillo-pharyngitis decreased from 26.6% in the period of 1998-2003 to 13.2% during 2004-2006, and alterations in the clonal composition of these isolates were reported [178, 180]. Based on a comparison of the clonal structure of macrolide-resistant and -susceptible isolates recovered in the same time period and geographical region, it has recently been shown that these two subpopulations of GAS are represented by distinct genetic lineages, presenting independent dynamics [177]. In the same study, macrolide resistance was found to be significantly associated with *emm* types 4, 22, and 28. Among invasive isolates, macrolide resistance also varies greatly throughout Europe. Scandinavian countries and Germany present the lowest resistance rates (1.5-5.5%), in contrast to Spain and Italy, which have the highest resistance levels (17-26.5%) [32, 85, 113, 131, 134, 164, 176]. Poland, Greece, and France have reported erythromycin resistance rates ranging from 8% to 12% [107, 185, 193]. In most of these studies, tetracycline resistance rates were higher than macrolide resistance, ranging from 6.1% in Norway to 46.3% in Poland [131, 193]. In Portugal, previously to this work only small collections of invasive GAS isolates had been studied, yielding resistance rates of 11% and 6% for erythromycin, and above 40% for tetracycline [132, 156].

Fluoroquinolone resistance is also frequently tested for epidemiological purposes, due to the emergence of GAS clones presenting reduced susceptibility to this class of antimicrobials, mostly associated with *emm* types 6, 11, and 75 [116, 210]. In Portugal, reduced fluoroquinolone susceptibility has been identified among isolates associated with oropharyngeal carriage, tonsillo-pharyngitis, and skin and soft tissue infections, frequently presenting *emm*6 [158]. Although active efflux may contribute to low-level resistance in *Streptococcus pneumoniae* and other viridians group streptococci, the most common mechanism of fluoroquinolone resistance in *Streptococcus* is the alteration of the target enzymes. Intermediate resistance is usually caused by first-step mutations in the quinolone resistance-determining region (QRDR) of the *parC* gene, encoding one of the subunits of topoisomerase IV. High level resistance usually requires a second-step mutation, most frequently in the QRDR of the *gyrA* gene, which encodes the DNA gyrase [82]. The spontaneous occurrence of such mutations is considered the main mechanism of emergence of

fluoroquinolone resistance, but evidence for horizontal transfer of the QRDR among streptococci has been reported, including between *S. pyogenes* and *S. dysgalactiae* [42, 155].

7.2. Potential GAS vaccines

Given the global economic burden associated with GAS infections, as well as the mortality and morbidity affecting patients with invasive GAS disease and post-streptococcal sequelae, there have long been efforts to develop an effective vaccine against GAS. In recent years, the advances in molecular biology, in particular the full-genome sequencing of several *S. pyogenes* genomes, allowed the identification of a growing number of potential vaccine targets [16, 65, 174]. Two broad categories of vaccines have been considered and developed to different extents: those based on conserved epitopes (like C5a peptidase, SpeB, SpyCEP, group A carbohydrate, fibronectin-binding proteins, SLS, SOF, and the C-terminal region of the M protein), and those based on variable surface antigens (like the N terminal region of the M protein and pili) [16, 35, 135, 198].

Currently, the most extensively developed vaccine, which has completed phase I and II clinical trials, is a 26-valent vaccine consisting of four recombinant fusion proteins, comprising a total of 26 distinct N-terminal M peptides and the streptococcal protective antigen (Spa) linked in tandem [35, 130]. This vaccine was designed based on epidemiological data from North America, including the M types most commonly responsible for invasive infections, pharyngitis, and all the serotypes known as "rheumatogenic". However, subsequent epidemiological data from several world regions indicated that the vaccine coverage might not be as high as initially predicted, especially in developing countries, where the incidence of acute rheumatic fever is higher [186]. A new formulation of the vaccine has recently been developed, including a total of 30 M peptides, which account for around 98% of pharyngitis cases in North America, 90% of invasive infections in the U.S.A., and 78% of invasive infections in Europe, according to recent estimates [36]. However, the actual vaccine coverage is hard to estimate and can be higher, since the 30-valent vaccine has been shown to evoke cross-opsonic bactericidal antibodies against several serotypes not included in the vaccine.

8. The *S. pyogenes* chromosome and the importance of horizontal gene transfer in GAS evolution

Since the publication of the first complete genome sequence of a GAS strain in 2001 [57], 19 other full genome sequences have been determined, annotated, and made publicly available, and at least another 39 are in project (http://www.ncbi.nlm.nih.gov/genome/genomes/175, accessed on March 27th 2013). The available completed genomes range between 1.75 Mb and 1.94 Mb in size and have a similar G+C content, around 38.5%. Remarkably, ~10% of the gene content is encoded on variably present exogenous genetic elements, mostly prophages, but also in integrated conjugative elements and plasmids [7, 138]. These MGEs and their related recombination events are thought to be the major contributors for genetic variation in GAS, which is observed even among strains of the same M serotype [129]. Horizontal gene transfer events have been shown to occur not only among GAS strains, but also between GAS and other streptococci [106]. Among other examples, there is evidence for the lateral transfer of *emm* and SAg genes between GAS and group G streptococci [92, 181], and the association of *emm*28 isolates with puerperal infections seems to be related to the acquisition of the RD2 DNA region from *S. agalactiae* [70].

Recombination events associated with MGEs can strongly contribute to the emergence of GAS clones with specific properties, like antimicrobial resistance or enhanced virulence, since these elements often carry antimicrobial resistance genes and virulence factors. In particular, several horizontal gene transfer events seem to have been involved in the emergence and dissemination of the highly invasive and successful M1T1 clone. These events included the sequential acquisition of prophages encoding the SAg SpeA2 and the DNase Sda1/SdaD2, as well as to the acquisition by homologous recombination of a 36 kb chromosomal region encoding NADase and SLO from an M12 strain, although the order by which these events occurred is not consensual [114, 190]. The production of the new SpeA variant (SpeA2) may have enabled the escape from herd immunity due to the absence of SpeA2-neutralizing antibodies in the host population prior to the emergence of this clone, besides contributing to virulence due to its superantigenic activity [114]. Sda1, on the other hand, may enhance the evasion to the host immune system by promoting the escape from NET entrapment [19]. Moreover, the presence of the *sda*1 gene has been shown to enhance the selection pressure for the acquisition of mutations in the CovR/S regulatory system that trigger the switch to an invasive phenotype [211]. The transfer of the 36 kb region from the

M12 genetic background, associated with the accumulation of spontaneous point mutations, was responsible for an increase in the expression of NADase and SLO, which have both been shown to contribute to GAS virulence [114, 190].

Similar recombination events have been suggested to be involved in the origin of an *emm*28 clone currently circulating in North America and Europe [70, 209], and in the evolution of *emm*3 isolates causing epidemics of invasive infections in Canada [8].

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AIMS OF THE THESIS

The epidemiological and molecular characterization of the *S. pyogenes* isolates causing invasive disease in different countries may offer important insights into the bacterial population dynamics underlying the resurgence of invasive GAS infections since the late 20th century, and ultimately contribute to improve the strategies used in the clinical management and prevention of these infections. Knowing the antimicrobial resistance rate of the circulating GAS population provides valuable information for assisting in the empirical treatment of GAS infections. The clonal structure and molecular properties of the isolates causing invasive infections are determinant for the potential efficacy of the vaccines based on variable GAS surface antigens, like the 30-valent M protein vaccine that is currently under development. On the other hand, it remains unclear if the reemergence of invasive GAS infections is associated with the rise of particular clones with enhanced virulence, while the role of variable bacterial factors, like the M protein or the streptococcal SAgs, in disease outcome remains not completely understood. Studies addressing these issues have yielded conficting results, but many of them were hampered by a limited genetic diversity of the studied isolates or by the use of insufficient typing methods.

In Portugal, prior to this work, scarce information about a very limited number of invasive GAS isolates had been reported in studies focusing primarily on isolates associated with pharyngitis and oropharyngeal carriage, with particular emphasis on macrolide resistance. Therefore, the present study aimed at characterizing in detail the GAS population associated with invasive infections in Portugal, in a non-outbreak context. On a first approach, a collection of 160 *S. pyogenes* isolates recovered from normally sterile sites in clinical laboratories distributed throughout the country during 2000-2005 were studied by serologic (T typing) and molecular methodologies (*emm* typing, PFGE macrorestriction profiling, MLST, and SAg gene profiling). The clonal relationships between the isolates were evaluated, and the molecular properties of the main lineages causing invasive GAS disease in Portugal were compared with the situation reported in other countries.

Another purpose that motivated the present studies was to test if the isolates causing invasive GAS infections in Portugal reflect the general circulating GAS population or if there are clones with an enhanced ability to cause invasive disease. In order to achieve this, and taking into consideration that the oropharynx is considered as the main reservoir of GAS isolates causing invasive infections in developed countries, the antimicrobial resistance and

molecular properties of the invasive isolates from 2000-2005 were compared with those of a collection of 320 isolates recovered from pharyngitis patients, in the same laboratories and during the same time period. A thorough statistical analysis of the data was performed, with the aims of identifying molecular properties, or pairwise combination of properties, that can be regarded as markers of enhanced or decreased invasiveness and of evaluating the interaction between those individual properties.

The use of a variety of typing methods to characterize a large collection (n = 480) of genetically and epidemiologically diverse *S. pyogenes* isolates allowed a comparison between the most common typing methods used for the characterization of GAS clones, regarding their discriminatory ability and congruence. A particular emphasis was given to SAg gene profiling, given the importance of SAg genes as markers for the presence and transfer of prophages, and the relevance of horizontal gene transfer mechanisms in the genetic diversification of *S. pyogenes*. The comparison of the SAg gene content of GAS isolates reported by different studies has been frequently hindered by a confusing gene nomenclature and by the occurrence of false positive or negative PCR results due to the use of unsuited primers. Therefore, one of the aims of the work presented in this thesis was to develop a PCR scheme capable of efficiently amplifying all the allelic variants of the 11 GAS SAg genes described to date, avoiding the amplification of nonfunctional gene remnants.

Finally, a collection of 191 invasive isolates recovered throughout Portugal between 2006 and 2009 were characterized using all the abovementioned methodologies and the results were compared with those obtained for the invasive strains isolated during 2000-2005. This work aimed to identify possible shifts in the clonal structure and antimicrobial resistance among the invasive GAS isolates in Portugal, as reported in other developed countries, as well as to evaluate the genetic diversification of the most prevalent lineages.

CHAPTER II

NONOUTBREAK SURVEILLANCE OF GROUP A STREPTOCOCCI CAUSING INVASIVE DISEASE IN PORTUGAL IDENTIFIED INTERNATIONALLY DISSEMINATED CLONES AMONG MEMBERS OF A GENETICALLY DIVERSE POPULATION

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SUMMARY

The typing of 160 invasive *Streptococcus pyogenes* isolates confirmed the importance of pulsed-field gel electrophoresis and multilocus sequence typing to define clones. The results identified an extremely diverse population and highlighted the importance of both internationally disseminated and local clones not previously associated with invasive disease.

A reemergence of invasive disease caused by *Streptococcus pyogenes* (a member of the group A streptococci [GAS]) has been noted since the late 1980s, both in North America and in Europe [3]. This increase in incidence of GAS infections has frequently been associated with specific clones, suggesting the possibility that the rise of particularly virulent clones was responsible for this reemergence. The identification of GAS clones in surveillance and epidemiological studies has frequently relied on serotyping using two variable surface antigens – the T antigen (T typing) and the M protein (M typing, or *emm* typing, as the protein is encoded by the *emm* gene) [3]. Recent work suggests that *emm* typing alone is not sufficient to unambiguously identify GAS clones and that this method must be complemented with an analysis by either pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) [1]. A total of 160 nonduplicate GAS isolates recovered from normally sterile sites (150 from blood, 7 from pleural fluid, and 3 from cerebrospinal fluid) were collected in 19 laboratories distributed throughout Portugal that were asked to submit all isolates between 2000 and 2005. The number of participating laboratories was not constant, and this variation was reflected in the number of isolates available in each of the study years: 6 in 2000 (3 laboratories), 15 in 2001 (8 laboratories), 17 in 2002 (10 laboratories), 27 in 2003 (12 laboratories), 39 in 2004 (15 laboratories), and 56 in 2005 (19 laboratories).

Twelve different T serotypes [20] were identified, and 23 isolates (14%) were non-typeable (Simpson's index of diversity [SID] ± 95% confidence interval [CI], 88.2% ± 2.3%) [1], whereas 30 different *emm* types were identified (SID ± 95% CI, 92.0% ± 2.0%). The presence of genes encoding GAS pyrogenic toxins was studied by PCR [2, 9, 16, 18]. As expected, the results confirmed the presence of the chromosomal genes *speB* and *speF* in all strains except one. Eleven different exotoxin gene profiles were identified (SID ± 95% CI, 82.2% ± 3.0%), but a significant fraction of the isolates (16%) were negative for all but the chromosomal genes (Table II).

The dendrogram based on the PFGE profiles of SmaI- or Cfr9I-digested total DNA [1, 19] of the isolates identified 13 major clusters accounting for 86% of all isolates (SID ± 95% CI, 92.0% ± 2.1%) (Figure II). Among the 37 isolates characterized by MLST [5], four new alleles were identified and submitted to the *S. pyogenes* MLST database, namely *recP*82, *gki*98, *gtr*73, and *murI*68, as well as seven new sequence types (STs; ST258 and ST406 to ST411), which are all single-locus variants of pre-existing STs, except for ST258, which is a double-locus variant of three preexisting STs. The overall level of correspondence between PFGE clusters and *emm* types was high, with a Wallace coefficient of 0.890, meaning that only 1 out of every 10 pairs of isolates grouped into the same PFGE cluster will not share the

same *emm* type [1]. In most cases, the presence of more than one *emm* type in the same lineage is associated with the presence of more than one ST. In two instances, although the isolates grouped by PFGE shared the same *emm* type, they presented different STs (PFGE clusters B and D) (Figure II and Table II). However, the two pairs of STs found among isolates expressing each of these *emm* types (ST15-ST406 and ST382-ST411) are single-locus variants of each other, supporting a close genetic relationship between the isolates as indicated by PFGE.

Globally, both *emm* type and PFGE cluster assignment were excellent predictors of the exotoxin gene profile, with Wallace coefficients of 0.906 and 0.871, respectively, such that any two isolates grouped together by these characteristics had a high probability (close to 90%) of sharing the same exotoxin gene profile.

In most countries, *emm* types 1, 3, and 28 have been traditionally associated with invasive GAS disease [4]. In this study, *emm* types 1 and 3 were also those expressed by isolates in the main PFGE clusters, A and B, which accounted for 30% of all isolates and were associated with ST28 and ST15-ST406, respectively. Several studies report a correlation between the presence of the *speA* gene and invasive GAS isolates, especially *emm*1 isolates causing streptococcal toxic shock syndrome [13, 22]. In the present study, *speA* was also detected in all isolates of the two major PFGE clusters, but all other isolates lacked *speA*, and the most frequent exotoxin gene found was *speC*. The exotoxin gene profile of the main invasive clone in Portugal is in agreement with previous reports for isolates of the same *emm* type (*emm*1) in other countries [2, 18, 23]. On the other hand, though *ssa* has recently been reported as being more prevalent among M1/*emm*1 invasive isolates than among M1/*emm*1 noninvasive isolates [4, 17], it was not found among any of the *emm*1 isolates in this study. Taken together with the high Wallace coefficient found for the PFGE clusters and the exotoxin gene profiles, this result suggests that there are two distinct lineages carrying the *emm*1 allele responsible for invasive infections and emphasizes the importance of using PFGE or MLST to fully characterize GAS clones [1].

Table II Properties of the PFGE clones of invasive *S. pyogenes* isolates from Portugal

PFGE cluster	No. of isolates (% of total)	T type[a]-*emm* type (no. of isolates)	ST[b] (no. of isolates)	Exotoxin gene profile[c]			
				speA	***speC***	***speH***	***ssa***
A	33 (20.6)	1-1 (31)	28 (2)	+	-[d]	-	-
		NT-1 (1)	28	+	-	-	-
		1-stIL103 (1)	28	+	-	-	-
B	15 (9.4)	3/13-3 (9)	**406**	+	-	-	+
		NT-3 (4)	15	+	-	-	+
		1-3 (2)	**406**	+	-	-	+
C	13 (8.1)	3/13-64 (5)	164 (2)	-	-	-	-
		NT-64 (4)		-	-	-	-
		1-64 (2)		-	-	-	-
		NT-53 (2)	11	-	+	-	-
D	12 (7.5)	6-6 (9)	382 (2)	-	+	-	-
		NT-6 (2)	**411**	-	+	-	-
		NT-18 (1)	402	-	-	-	-
E	11 (6.9)	12-12 (10)	36	-	-[e]	+	-
		B3264-94 (1)	89	-	-	+	-
F	10 (6.3)	B3264-89	**408** (2)	-	+[f]	-	-
G	8 (5.0)	28-28	52 (2)	-	+	-	-
H	8 (5.0)	4-4	39 (3)	-	+	-	+
I	7 (4.4)	5/27/44-44/61 (6)	25	-	-	+	+
		4-4 (1)	39	-	+	-	+
J	6 (3.8)	3/13-43 (2)	3	+	-	-	-
		NT-43 (2)	3	+	-	-	-
		NT-58 (2)	**410**	-	+	+	-
K	5 (3.1)	11-78 (3)	**409**	-	+	-	-
		3/13-78 (1)	**409**	-	+	-	-
		11-89 (1)	**407**	-	-	-	-
L	5 (3.1)	28-28 (3)	52	-	+	-	-
		B3264-89 (2)	101	-	+	-	-
M	5 (3.1)	9-74 (1)	120	-	+	-	-
		12-22 (1)	46	-	+	-	+
		NT-58 (1)		-	-	-	-
		4-st106M (1)		-	-	-	-
		NT-9 (1)		-	-	-	-
Other[g]	22 (13.8)	15 different combinations[h]	Various[i]	7 different profiles[j]			

[a] NT, nontypeable.
[b] Numbers in bold indicate STs described for the first time in this report. The STs of at least two representatives of each PFGE cluster were determined. When more than one *emm* or T type was present in the same clone, isolates expressing different surface antigens were selected.
[c] All isolates were positive for *speB* and *speF*, except one T28-*emm*28 isolate in clone L, from which only the *speC* gene was amplified by PCR.
[d] One isolate was positive for the *speC* gene.
[e] Three isolates were positive for the *speC* gene.
[f] One isolate was negative for the *speC* gene.
[g] Isolates with unique PFGE patterns or included in clusters of fewer than five isolates.
[h] Of the 15 different T type-*emm* type combinations, the following were not identified in any of the main lineages: 13-113 (three isolates), 11-11 and 28-87 (two isolates each), and 13-77, 13-114, 13-103, NT-stG1750, 3/13-30, 9-9, 1-22, 1-90, NT-11, and 2-2 (one isolate each).
[i] Two isolates, one ST52 (T28-*emm*28) and the other ST258 (NT-stG1750), were analyzed.
[j] Of the 7 different profiles, the following was not identified in any of the main lineages: negative for *speA*, *speC*, and *speH* and positive for *ssa*.

When we examined the association of the major PFGE clusters with the age groups of the patients, only the distribution of PFGE cluster E among isolates from the three age groups considered (≤18 years, 18 to 60 years, and >60 years) was significantly different from the expected distribution, with this cluster assignment being more frequent among isolates from pediatric patients than among those from adults (P = 0.003; one-tailed Fisher's exact test) [10]. Although *emm*12, the dominant type in this PFGE cluster, corresponded to the most abundant M type found among healthy children in Tokyo during an extensive longitudinal study [8], an association between M12 and pediatric invasive disease was not previously noted. The fact that the isolates described here were not geographically or temporally clustered suggests that a clone defined by a characteristic PFGE profile, ST36, and carrying the *emm*12 allele is associated with children and pediatric invasive disease in particular.

To evaluate the geographic spread of the clones identified we resorted to the data available in the *S. pyogenes* MLST database (http://spyogenes.mlst.net). Representatives of ST28 are widely dispersed, being found in several countries worldwide, while ST15, associated with the B PFGE cluster, has been found only in Europe and North America. The isolates exhibiting *emm*28, the third-most-frequent *emm* type in this and most studies of invasive GAS disease, were dispersed among several PFGE clusters, with macrolide-resistant isolates grouping together and apart from susceptible isolates (data not shown), suggesting that there may be other genetic differences between these isolates. However, the PFGE distinction was not supported by the other methods used to characterize them, with all isolates presenting ST52 and identical exotoxin profiles (Table II). ST52 is also widely geographically dispersed, being found in several countries worldwide.

In contrast, the STs found in the other two largest PFGE clusters, C (ST164 and ST11) and D (ST382, ST411, and ST402), have a much more limited geographic distribution. ST164 has been found in Israel, and ST11 has been found in Trinidad and the United States, while ST382 has been found in Spain, Russia and Austria, and ST411 and ST402 have been limited to Portugal. Moreover, none of these STs were previously found to cause invasive infections outside of Portugal. Interestingly, the ST11 isolates found in the MLST database were associated exclusively with impetiginous lesions, while the ST11 isolates reported here were recovered from blood. Several lines of evidence suggest that invasive isolates in industrialized countries reside mainly in a throat reservoir [7]. Although the identification in this study of macrolide-resistant isolates representing the major clones found in isolates causing pharyngitis [19] is in agreement with this suggestion (data not shown), the data

available regarding ST11 isolates recovered in other countries suggest that strains with this ST may reside in another important GAS reservoir, the impetiginous lesion.

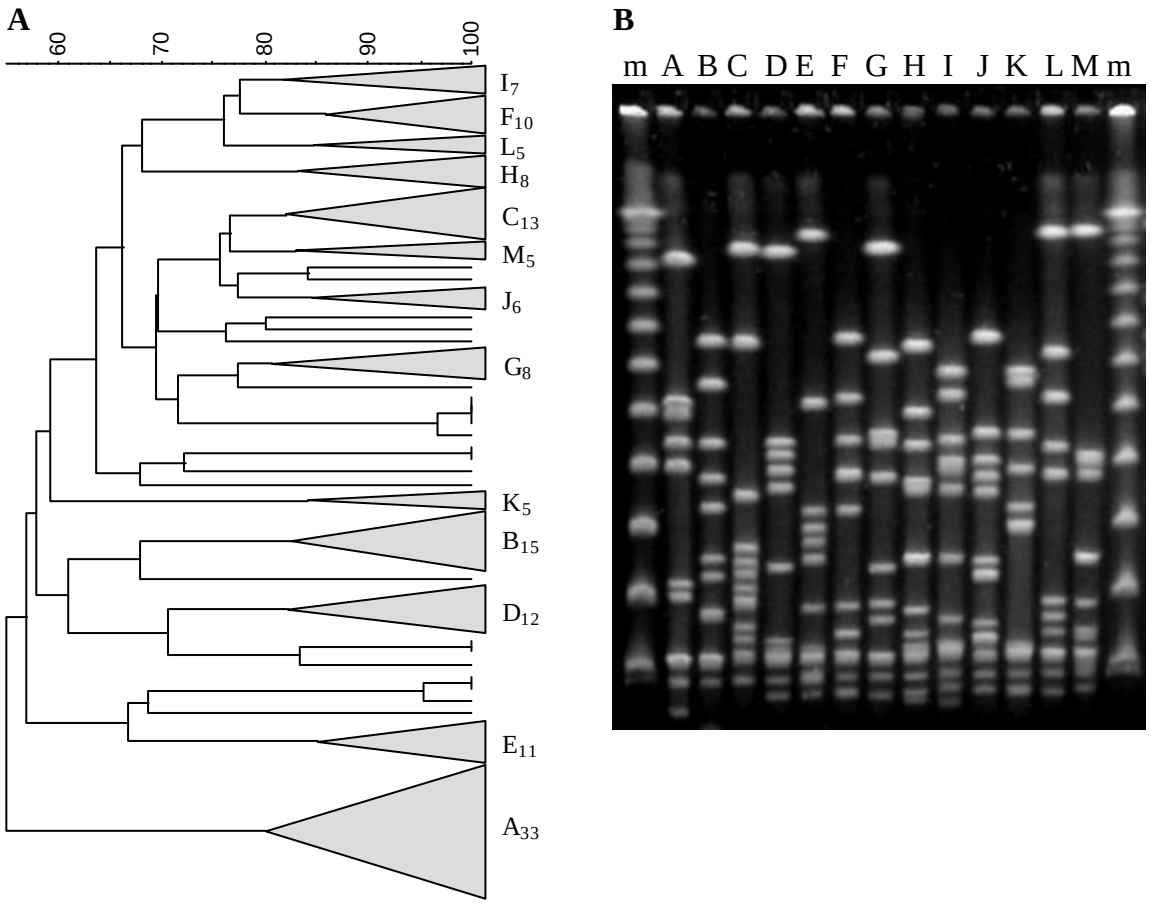

Figure II PFGE SmaI/Cfr9I macrorestriction profile analysis of *S. pyogenes* isolates from invasive infections in Portugal. (A) Dendrogram showing a cluster analysis of the PFGE profiles of the 160 isolates studied as determined by the unweighted-pair group method using average linkages. Dice coefficients (percentages) are indicated in the scale above the dendrogram. Each major clone (defined as a group of ≥ 5 isolates with a Dice coefficient of ≥ 80%) is represented by a triangle with a size proportional to the number of isolates included in the cluster, followed by a capital letter designating the clone and a subscript number indicating the number of isolates it comprises. (B) PFGE profiles of representatives of each clone. Capital letters above the lanes correspond to the clone designations; m, lambda ladder PFGE marker (New England Biolabs, Beverly, MA).

While most isolates presented *emm* alleles associated exclusively with *S. pyogenes*, one isolate had an *emm* allele (stG1750) that has been identified only among group G streptococci (GGS), according to the Centers for Disease Control and Prevention database. The MLST analysis of the stG1750 isolate revealed a novel allelic profile which was assigned ST258, but all alleles at each of the genes had already been found in other STs present in the *S. pyogenes* MLST database. An eBURST analysis [6] identified this ST as a singleton, but there are three double-locus variants in the *S. pyogenes* MLST database. Taken together, the results of molecular typing support the identification of this isolate as *S. pyogenes*, raising the

possibility that it may have acquired the *emm* gene from GGS by lateral gene transfer. Evidence for the horizontal transfer of *emm* genes between GAS and GGS has been reported previously [21], but this *emm* type was not found among a large collection of group G and C streptococci responsible for human infections in Portugal during the same time period as the isolates analyzed in this report [14].

Previous studies have addressed specific questions regarding GAS infections in Portugal [12, 15, 19], but this is the largest and more detailed study of GAS invasive isolates from Portugal, allowing the characterization of the main lineages of *S. pyogenes* causing invasive disease. This study identified extensive diversity among GAS isolates, as attested by the high SIDs of all typing methodologies, that results in a lower potential coverage of GAS invasive infections in Portugal by a future 26-valent vaccine (69%) than in the United States [11].

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CHAPTER III

GROUP A STREPTOCOCCI CLONES ASSOCIATED WITH INVASIVE INFECTIONS AND PHARYNGITIS IN PORTUGAL PRESENT DIFFERENCES IN *EMM* TYPES, SUPERANTIGEN GENE CONTENT AND ANTIMICROBIAL RESISTANCE

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SUMMARY

A few lineages of Group A *Streptococci* (GAS) have been associated with a reemergence of severe invasive streptococcal disease in developed countries. However, the majority of the comparisons between invasive and non-invasive GAS isolates have been performed for collections of reduced genetic diversity or relied on limited typing information to distinguish clones. We characterized by several typing methods and compared a collection of 160 isolates recovered from normally sterile sites with 320 isolates associated with pharyngitis and recovered in the same time period in Portugal. Although most of the isolates belonged to clones that were equally prevalent in invasive infections and pharyngitis, we identified markers of invasiveness, namely the *emm* types 1 and 64, and the presence of the *speA* and *speJ* genes. In contrast, *emm*4, *emm*75, and the *ssa* and *speL/M* genes were significantly associated with pharyngitis. There was a strong agreement between the *emm* type, the superantigen (SAg) genes and the clusters defined by pulsed-field gel electrophoresis (PFGE) profiling. Therefore, combinations of particular *emm* types and SAg genes frequently co-occurred in the same PFGE cluster, but there was no synergistic or antagonistic interaction between them in determining invasiveness. Only macrolide-susceptible PFGE clones were significantly associated with invasive infections or pharyngitis, while the clones of resistant isolates sharing all other molecular properties analyzed were equally prevalent in the two groups of isolates. This study confirmed the importance of the widely disseminated *emm*1-T1-ST28 clone in invasive infections but also identified other clones linked to either invasive infections (*emm*64-ST164) or pharyngitis (*emm*4-T4-ST39), which may be more limited in their temporal and geographical spread. Clonal properties like some *emm* types or SAg genes were associated with disease presentation, highlighting the importance of bacterial genetic factors to the outcome of GAS infections, although other, yet unidentified factors may also play an important role.

INTRODUCTION

Streptococcus pyogenes (Lancefield group A *Streptococcus*, GAS) remains one of the most common human pathogens, being responsible for uncomplicated superficial infections of the respiratory tract and skin, such as tonsillo-pharyngitis and impetigo, but also causing severe and rapidly progressing invasive disease such as necrotizing fasciitis, bacteremia, streptococcal toxic shock syndrome (STSS), puerperal sepsis, pneumonia, and meningitis [8]. Although the incidence and severity of GAS infections in industrialized countries decreased for most of the 20th century, a reemerge of GAS invasive disease has been noted since the late 1980s, both in North America and in Europe [11]. The annual incidence of GAS invasive disease has been estimated at 2.45/100 000 for developed countries, with a median case fatality rate of 15% [4].

The increase in the incidence of GAS invasive infections has been frequently associated with specific clones, raising the possibility that the rise of particularly virulent clones was responsible for this reemergence, in particular the M1T1 clone which is dominant among invasive GAS isolates in most developed countries [23, 30]. However, a higher representation of a particular clone in invasive infections may be simply due to a high prevalence of that same clone in the general GAS population. To address this question several studies have performed comparisons between the characteristics of the invasive clones and the *S. pyogenes* isolates associated with carriage or uncomplicated infections in the same time period and geographic region. Some of those studies reported a differential distribution of M/*emm* types or of T types between invasive and non-invasive isolates and confirmed an association between serotype T1 and M1/*emm*1 and invasive infection [7, 12, 27, 42], but many others found that the major clones responsible for invasive infections had a similar prevalence among non-invasive infections [10, 33, 34]. However, most of the studies performing such comparisons were either restricted to small numbers of isolates or were limited in the typing methodologies used, relying essentially on M/*emm* typing.

Serotyping of GAS based on protein M, a major surface virulence factor, has long been used as the gold standard for the epidemiological surveillance of the infections caused by this pathogen. In recent years it has been widely replaced by an equivalent approach based on sequencing the hypervariable region of the *emm* gene encoding the M protein. However, recent studies show that *emm* typing alone is not sufficient to unambiguously identify GAS clones and that it must be complemented with other typing methods such as pulsed-field gel

electrophoresis (PFGE) macrorestriction profiling or multilocus sequence typing (MLST) [5]. Streptococcal superantigens (SAgs) secreted by *S. pyogenes* play an important role in the pathogenesis of the infections caused by this species [38]. The profiling of the eleven SAg genes described so far (*speA*, *speC*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *ssa*, *smeZ*) can be used as a typing methodology [35]. Some studies suggested an association between the presence of certain SAg genes or of certain SAg gene profiles and invasive infections [10, 22], although others failed to establish such an association, reporting instead a strong link between the SAg profile and the *emm* type, regardless of the isolation site [34, 35].

We have previously characterized a collection of 160 invasive GAS isolates collected throughout Portugal between 2000 and 2005, and found a very high genetic diversity among this collection, but with a dominant clone representing more than 20% of the isolates, which was characterized as *emm*1-T1-ST28 and carried the gene *speA* [18]. The aim of the present study was to evaluate if the clone distribution among the invasive GAS isolates in Portugal reflected the clonal structure of the isolates causing pharyngitis, in terms of molecular properties and antimicrobial resistance. In order to do that, 320 non-duplicate isolates collected from pharyngeal exudates associated with tonsillo-pharyngitis in the same time period were studied by *emm* typing, T typing, SAg profiling, PFGE macrorestriction profiling, and selected isolates were also submitted to MLST analysis. All isolates were also tested for their susceptibility to clinically and epidemiologically relevant antimicrobial agents. The great majority of the clones were found with a similar frequency among invasive infections and pharyngitis. Still, some clones were shown to have a higher invasive disease potential and it was also possible to establish significant associations between some *emm* types and SAg genes and disease presentation.

MATERIALS AND METHODS

Bacterial isolates

The invasive isolates (*n* = 160) were collected from normally sterile sites, and their partial characterization was previously reported [18]. A total of 320 non-duplicate GAS isolates were randomly selected among a collection of 1604 isolates recovered from pharyngeal exudates of patients presenting with tonsillo-pharyngitis in 32 laboratories distributed throughout Portugal, between 2000 and 2005, in the proportion of 1:2 (invasive:pharyngitis) for each studied year. These isolates were recovered from pediatric patients (<18 yrs) and showed a balanced distribution by gender. The subset of macrolide-resistant pharyngeal isolates had been partially characterized [36, 37]. Strains were identified by the submitting laboratories and confirmed in our laboratory by colony morphology, β-hemolysis and the presence of the characteristic group antigen (Slidex Strepto A, BioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Susceptibility tests were performed by disk diffusion on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) using the following antibiotic disks (Oxoid, Basingstoke, UK): penicillin, vancomycin, erythromycin, tetracycline, levofloxacin, chloramphenicol, clindamycin, quinupristin/dalfopristin, and linezolid. Whenever isolates with intermediate susceptibility were identified, the results were confirmed by MIC determination using E-test strips (BioMérieux, Marcy l'Etoile, France). The macrolide resistance phenotype was determined as previously described [25]. Susceptibility to bacitracin was determined for all isolates using disks containing 0.05 U of bacitracin (Oxoid, Basingstoke, UK), as described elsewhere [37].

Detection of antimicrobial resistance genetic determinants

Bacterial DNA was prepared according to the protocols of the Centers for Disease Control and Prevention (CDC). Determination of the macrolide resistance genotype was performed for strains presenting either the M or the MLS_B macrolide resistance phenotype, by a multiplex PCR reaction with primers to detect the *erm*(B), *erm*(A) and *mef* genes, as

previously described [14]. Isolates carrying the *mef* gene were subjected to a second PCR reaction in order to discriminate between *mef*(A) and *mef*(E) [36]. Tetracycline resistant isolates were PCR-screened for the presence of the genes *tet*(K), *tet*(L), *tet*(M), and *tet*(O) as previously described [41]. Strains harboring each of the resistance genes were used as positive controls for the PCR reactions.

T-typing

Strains were cultured in Todd-Hewitt broth (Oxoid, Basingstoke, UK) at 30ºC overnight and treated with swine pancreatic extract, using the Auxiliary Reagents for Hemolytic Streptococcus Typing (Denka Seiken, Tokyo, Japan), and following the manufacturer's instructions. T serotypes were determined by slide agglutination with 5 polyvalent and 19 monovalent sera (Hemolytic Streptococcus Group-A Typing Sera, Denka Seiken).

***emm*-typing and SAg gene profiling**

The *emm*-typing of all isolates was performed according to the protocols and recommendations of the CDC, and the first 240 bases of each sequence were searched against the *emm* CDC database. Identity of ≥ 95% with previously described sequences over the 150 bases considered allowed the assignment of an *emm* type. The presence of the SAg genes *speA*, *speC*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *smeZ*, and *ssa*, and of the chromosomally encoded exotoxin genes *speB* and *speF* (used as positive control fragments) was assessed in all 160 invasive and 320 non-invasive GAS isolates by two multiplex PCR reactions as described elsewhere [17].

PFGE macrorestriction profiling and MLST

Agarose plugs of bacterial DNA were prepared as previously described [37]. After digestion with SmaI or Cfr9I (Fermentas, Vilnius, Lithuania), the fragments were resolved by PFGE [37]. The isoschizomer Cfr9I was used only for the isolates with the M phenotype, which were not digested by SmaI [5, 37]. The macrorestriction patterns generated were compared using the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) to create UPGMA (unweighted pair group method with arithmetic mean) dendrograms. The Dice similarity coefficient was used, with optimization and position tolerance settings of 1.0

and 1.5, respectively. PFGE clones were defined as groups of >5 isolates presenting profiles with ≥ 80% relatedness on the dendrogram [5]. MLST analysis was performed as described elsewhere [13] for representatives of each PFGE cluster (a total of 100 non-invasive and 70 invasive isolates). When more than one *emm* or T-type was present in the same PFGE cluster, isolates expressing different surface antigens were selected. Allele and sequence type (ST) identification was performed using the *S. pyogenes* MLST database. Whenever new alleles were identified, chromatograms of both strands were submitted to the database curator for approval and an allele number was assigned. Clonal complexes were determined using the goeBURST algorithm implemented in PHYLOViZ [16].

Statistical analysis

The diversities of the different PFGE clusters were compared using the Simpson's index of diversity (SID) with corresponding 95% confidence intervals ($CI_{95\%}$) [5]. Differences in antibiotic resistance between the invasive and non-invasive groups of isolates were evaluated using Fisher's exact test. *P* values < 0.05 were considered to indicate statistical significance. SAg genes, *emm* types and PFGE types were screened for associations with the invasive group by computing an odds-ratio and an associated Fisher's exact test. Additionally, pairs of individual SAg genes with each other or with *emm* types or PFGE types were similarly tested for the association of each pairs' co-occurrence with the invasive group of isolates. For the pairs where at least one of the types individually or their co-occurrence were associated (either positively or negatively) with the invasive group, two more tests were done, to investigate if the association of one of the types individually was modified by the co-occurrence of the other type in the pair (synergism or antagonism). Considering a pair of types A and B, this test compares the proportion of invasive isolates among the ones that have A type but not B with the same proportion among isolates that have both A and B types. If the proportions are statistically different, according to a Fisher's exact test, we can conclude that type B modifies the association of type A with the invasive group of isolates. Conversely, if the proportion of invasive isolates among the ones that have the B type but not A differs from the same proportion among isolates that have both A and B types, type A modifies the association of type B with the invasive group. If the isolates that are simultaneously of the A and B type show a significantly stronger association with invasive infection than the one observed for isolates having either the A or B type, the types are said to be synergistic. If, on the other hand, isolates that are simultaneously of the A and B type show a significantly

weaker association with invasive infection than the one observed for isolates having either the A or B type, the types are said to be antagonistic. All the p-values obtained in each step of the screening procedure were corrected for multiple testing through the False Discovery Rate (FDR) linear procedure [3].

RESULTS

Antimicrobial resistance

All isolates were fully susceptible to penicillin, quinupristin/dalfopristin, chloramphenicol, vancomycin, linezolid, and levofloxacin (Table III-1). Among the invasive isolates, 19 (12%) were resistant to erythromycin, while the isolates associated with pharyngitis presented significantly higher macrolide resistance – 21% (P = 0.016, two-tailed Fisher's exact test). Among the invasive macrolide-resistant isolates, 10 (53%) presented the M phenotype and were therefore susceptible to clindamycin, whereas the remaining nine (47%) were also constitutively resistant to clindamycin ($cMLS_B$ phenotype). The proportion of the two phenotypes was similar among the pharyngitis isolates, with 37 isolates (55%) presenting the M phenotype and 30 (45%) presenting the MLS_B phenotype (one with inducible resistance and the others with constitutive resistance to clindamycin). All the isolates presenting the M phenotype of macrolide resistance carried only the *mef*(A) variant of the *mef* determinant. The $cMLS_B$ isolates carried only the *erm*(B) gene, except for one pharyngitis isolate which also harbored *mef*(A), and the only $iMLS_B$ isolate in the collection that presented the *erm*(A) gene.

In contrast to erythromycin, tetracycline resistance was much lower among the pharyngitis isolates when compared with the invasive group (6% vs 17%, $P < 0.001$). One invasive isolate presented intermediate resistance to tetracycline (MIC = 3μg/ml). All the resistant strains carried the *tet*(M) gene, except one pharyngitis isolate for which no PCR product was obtained for any of the screened tetracycline-resistance genes. The *tet*(L) gene was detected in only one pharyngitis isolate, which also harbored *tet*(M), while the genes *tet*(K) and *tet*(O) were not amplified in any of the studied isolates. Overall there was a positive association between the genes *tet*(M) and *erm*(B) ($P < 0.001$), but that association was not observed among the subset of invasive isolates, since only three of the 27 *tet*(M)-positive invasive isolates also carried the *erm*(B) gene ($P = 0.178$).

Bacitracin resistance was detected in a total of 23 isolates (5%), with no significant differences among the two types of infection considered. All these isolates expressed the $cMLS_B$ phenotype of macrolide resistance and were tetracycline-susceptible.

Table III-1 PFGE clusters presenting antimicrobial resistant isolates collected from invasive infections and pharyngitis in Portugal.

PFGE cluster[a]	Antimicrobial resistance[b]	No. of resistant isolates	
		Invasive	Pharyngitis
C_{38}	Tet		1
D_{36}	MLS_B		1
	M		1
G_{27}	M	6	19
	M,Tet	1	
H_{26}	MLS_B,Bac	6	17
	Tet		1
I_{24}	MLS_B,Tet		1
J_{16}	Tet	12	1
K_{14}	M		1
L_{13}	MLS_B,Tet	1	6
	Tet	2	
M_{11}	MLS_B,Tet	1	
N_{10}	Tet	1	1
	MLS_B,Tet		1
O_9	M	4	5
R_6	M		3
S_6	M		1

[a]Clusters are designated by capital letters and a subscript number indicating the number of isolates in each cluster.

[b]The antibiotics tested were penicillin quinupristin/dalfopristin, chloramphenicol, vancomycin, linezolid, levofloxacin, erythromycin, clindamycin, tetracycline, and bacitracin. M, presenting the M phenotype of macrolide resistance; MLS_B, presenting the MLS_B phenotype of macrolide resistance; Tet, non-susceptibility to tetracycline; M,Tet, presenting the M phenotype of macrolide resistance and resistance to tetracycline; MLS_B,Tet, presenting the MLS_B phenotype of macrolide resistance and resistance to tetracycline; MLS_B,Bac, presenting the MLS_B phenotype of macrolide resistance and resistance to bacitracin.

Characterization of GAS clones

Globally, among the 480 isolates there were 36 *emm* types, 17 T types, and 49 SAg profiles (the genes included in each SAg profile are presented in Table III-S). In the subset of 170 isolates (100 from pharyngitis and 70 from invasive infections) selected for MLST analysis, 49 different STs were identified. Nineteen PFGE clusters (groups of > 5 isolates presenting ≥ 80% similarity on the PFGE profile) were obtained including 268 pharyngitis isolates and 143 invasive isolates (86% of all isolates) (Table III-2 and Table III-3). Except for R_6, isolates grouped into PFGE clusters presented some variability in their *emm* type, ST, T type, or SAg profile, with most variability found in the later two properties. Still, in most PFGE clusters the majority of the isolates were characterized by a single profile of dominant properties. The *emm* diversity among the PFGE clusters differed significantly (Table III-4). Within each PFGE cluster, different *emm* types were associated with distinct SAg profiles (Table III-2 and Table III-3), although globally the *emm* and PFGE had a similar predictive power over the SAg profile (data not shown).

Table III-2 Properties of the PFGE clusters with >15 GAS isolates collected from invasive infections and tonsillo-pharyngitis in Portugal.

PFGE cluster[a]	*emm* type	No. of isolates (% of total)		T type[b] (no. of isolates)	SAg genes profile (no. of isolates)	ST[c] (no. of isolates)
		Invasive	Pharyngitis			
A_{51}	3	15 (9.4)	36 (11.25)	3 (22), NT (14), 3/13 (13), 1 (2)	8 (48), 37 (2), 2 (1)	406 (8), 15 (4), 315 (2)
B_{49}	1	28 (17.5)	20 (6.3)	1 (46), NT (2)	10 (47), 3 (1)	28 (10)
	stIL103	1 (0.6)	0	1 (1)	10 (1)	28 (1)
C_{38}	89	12 (7.5)	25 (7.8)	B3264 (37)	27 (21), 29 (8), 46 (5), 43 (2), 40 (1)	408 (5), 553 (1), 101 (2)
	75	0	1 (0.3)	25 (1)	42 (1)	150 (1)
D_{36}	12	10 (6.3)	25 (7.8)	12 (29), NT (6)	33 (29), 16 (5), 46 (1)	36 (13), 551 (2)
	94	1 (0.6)	0	B3264 (1)	35 (1)	89 (1)
E_{30}	6	11 (6.9)	19 (5.9)	6 (27), NT (2), 2(1)	2 (28), 5 (1), 9 (1)	382 (6), 411 (3)
F_{29}	4	1 (0.6)	28 (8.8)	4 (29)	23 (27), 22 (2)	39 (5)
G_{27}	4	8 (5.0)	19 (5.9)	4 (23), B3264 (2), 2/27/44 (1), 2/4 (1)	23 (23), 30 (2), 40 (1), 41 (1)	39 (8), 561 (1)
H_{26}	28	7 (4.4)	17 (5.3)	28 (23), NT (1)	27 (13), 24 (10), 15 (1)	52 (10)
	22	0	1 (0.3)	12 (1)	3 (1)	nd
	75	0	1 (0.3)	NT (1)	7 (1)	481 (1)
I_{24}	44/61	6 (3.8)	16 (5.0)	2/27/44 (19), NT (2), 12 (1)	32 (16), 12 (6)	25 (5), 554 (1)
	75	0	1 (0.3)	25 (1)	36 (1)	150 (1)
	89	0	1 (0.3)	5/27/44 (1)	6 (1)	555 (1)
J_{16}	64	11 (6.9)	0	3/13 (5), NT (4), 1 (2)	46 (10), 43 (1)	164 (4), 124 (1)
	53	2 (1.3)	0	NT (2)	26 (2)	11 (1)
	74	0	1 (0.3)	B3264 (1)	11 (1)	120 (1)
	87	0	1 (0.3)	28 (1)	38 (1)	62 (1)
	89	0	1 (0.3)	B3264 (1)	43 (1)	568 (1)

[a] Clusters are designated by capital letters and a subscript number indicating the number of isolates in each cluster.
[b] NT, non-typeable.
[c] nd, not determined.

Table III-3 Properties of the PFGE clusters with <15 GAS isolates collected from invasive infections and tonsillo-pharyngitis in Portugal.

PFGE cluster[a]	*emm* type	No. of isolates (% of total)		T type[b] (no. of isolates)	SAg genes profile (no. of isolates)	ST[c] (no. of isolates)
		Invasive	Pharyngitis			
K_{14}	2	1 (0.6)	13 (4.1)	2 (13), 4 (1)	31 (12), 48 (2)	55 (5)
L_{13}	22	1 (0.6)	7 (2.2)	12 (8)	21 (6), 13 (1), 19 (1)	46 (2), 389 (1)
	9	1 (0.6)	1 (0.3)	9 (1), NT (1)	46 (2)	75 (2)
	2	0	1 (0.3)	2 (1)	31 (1)	55 (1)
	74	1 (0.6)	0	9 (1)	5 (1)	120 (1)
	st106M	1 (0.6)	0	4 (1)	49 (1)	53 (1)
M_{11}	28	8 (5.0)	3 (0.9)	28 (11)	24 (7), 27 (3), 15 (1)	52 (5)
N_{10}	87	2 (1.3)	7 (2.2)	28 (8), 6 (1)	20 (3), 27 (3), 2 (1), 18 (1), 44 (1)	62(2)
	22	0	1 (0.3)	12 (1)	21 (1)	46 (1)
O_9	1	4 (2.5)	5 (1.6)	1 (8), 13 (1)	10 (9)	28 (4)
P_8	78	4 (2.5)	4 (1.3)	11 (7), 3/13 (1)	29 (8)	409 (3)
Q_8	43	4 (2.5)	0	3/13 (2), NT (2)	11 (4)	3 (2)
	58	2 (1.3)	2 (0.6)	NT (4)	17 (3), 14 (1)	410 (3), 176 (1)
R_6	75	0	6 (1.9)	25 (6)	39 (6)	150 (2)
S_6	9	1 (0.6)	4 (1.3)	9 (5)	40 (5)	75 (2)
	12	0	1 (0.3)	12 (1)	33 (1)	36 (1)

[a] Clusters are designated by capital letters and a subscript number indicating the number of isolates in each cluster.
[b] NT, non-typeable

Unrelated STs within the same PFGE clusters were associated with isolates of different *emm* types, while isolates of the same *emm* type presented the same ST or single-locus variants (SLVs) (Table III-2 and Table III-3). The only exceptions were ST39 and ST561 that were both associated with cluster G_{27} and *emm*4, but were double-locus variants (DLVs) of each other. In clone I_{24}, four distinct STs were found. While ST25 and ST554 were SLVs and were both associated with *emm*44/61, ST150 belonged to a different clonal complex, but was also associated with a different *emm* type (*emm*75). Finally, ST555 despite being associated with an isolate of a different *emm* type (*emm*89) is a SLV of ST25, which may explain why this isolate was clustered in I_{24} and not in the major PFGE cluster associated with this *emm* type (C_{38}).

Isolates expressing the M phenotype of macrolide resistance belonged mostly to clusters G_{27}, O_9, and R_6, while the majority of MLS_B isolates were clustered in H_{26} and L_{13} (Table III-1). Isolates belonging to *emm* types 1, 4, and 28 were separated into different PFGE clusters according to macrolide resistance (B_{49} and O_9 for *emm*1; F_{29} and G_{27} for *emm*4; H_{26} and M_{11} for *emm*28, respectively), while the remaining characteristics (T type, ST, and SAg profile) of each pair of PFGE clusters were the same.

Bacitracin-resistant isolates were all clustered in PFGE H_{26} and were characterized as *emm*28-T28, except for one isolate that was *emm*22-T12. However, this cluster was not restricted to bacitracin-resistant isolates, since it also included three bacitracin susceptible isolates, two of which were also *emm*28-T28, while the other was *emm*75 but T non-typeable.

Table III-4 Simpson's index of diversity and 95% confidence intervals ($CI_{95\%}$) of *emm* types for each PFGE cluster.

PFGE cluster[a]	**No. *emm* types**	**SID [$CI_{95\%}$]**
B_{49}	2	0.041 [0-0.118]
C_{38}	2	0.053 [0-0.151]
D_{36}	2	0.056 [0-0.159]
H_{26}	3	0.151 [0-0.336]
I_{24}	3	0.163 [0-0.361]
J_{16}	5	0.533 [0.255-0.812]
L_{13}	5	0.628 [0.353-0.903]
N_{10}	2	0.200 [0-0.504]
Q_8	2	0.571 [0.571-0.571]
S_6	2	0.333 [0-0.739]

[a] PFGE clusters A_{51}, E_{30}, F_{29}, G_{27}, K_{14}, M_{11}, O_9, P_8, and R_6 include only one *emm* type (SID=0).

Surface antigen differences between invasive and pharyngitis isolates

The invasive isolates were significantly less diverse than the pharyngitis isolates by T typing and SAg profiling (Table III-5). However, while the *emm* type distribution varied between the invasive and pharyngitis isolates ($P < 0.001$) no differences were noted in the T types. Sixteen *emm* types occurred only in invasive infection or pharyngitis, but in most cases the small number of isolates associated with these *emm* types prevented the differences from reaching statistical significance (Figure III-1). In contrast, the overrepresentation of *emm* types 1, 4, 64, and 75 in one of the groups was statistically supported.

Table III-5 Simpson's index of diversity and 95% confidence intervals ($CI_{95\%}$) of the typing methods used in the analysis of the 160 invasive isolates and 320 pharyngitis isolates.

Typing method	Invasive		Pharyngitis	
	No. types	SID [$CI_{95\%}$]	No. types	SID [$CI_{95\%}$]
T typing	13	0.882 [0.859-0.904]	17	0.915 [0.907-0.923]
emm typing	30	0.920 [0.900-0.940]	26	0.921 [0.911-0.931]
PFGE profiling	30	0.930 [0.912-0.947]	44	0.947 [0.939-0.954]
SAg profiling	27	0.911 [0.891-0.931]	46	0.941 [0.932-0.951]

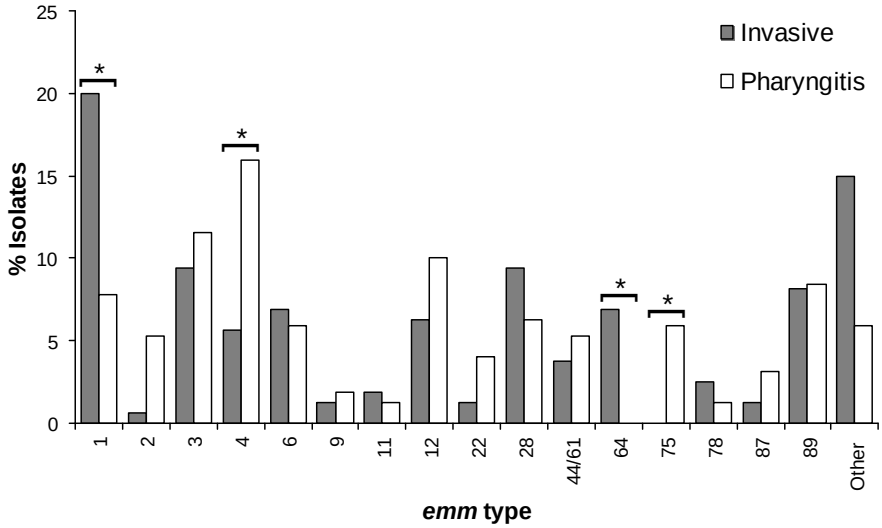

Figure III-1 Distribution of *emm* types among 160 invasive isolates and 320 pharyngitis isolates. Other includes *emm* types identified in ≤ 5 isolates - *emm*18 (*n*=4), 25 (*n*=1), 29 (*n*=2), 30 (*n*=1), 43 (*n*=4), 48 (*n*=1), 53 (*n*=3), 58 (*n*=5), 74 (*n*=2), 77 (*n*=4), 90 (*n*=1), 94 (*n*=3), 102 (*n*=3), 103 (*n*=1), 113 (*n*=3), 114 (*n*=1), 118 (*n*=1), *st*106M (*n*=1), *st*G1750 (*n*=1), *st*IL103 (*n*=1). The asterisk indicates significant differences ($P<0.001$).

SAg differences between invasive and pharyngitis isolates

A detailed analysis of the SAg profiling results of the isolates is performed elsewhere [17]. Briefly, the chromosomally encoded SAg genes *smeZ* and *speG* were the most frequent among the 480 isolates (*n* = 461 and 417, respectively), followed by *speC* (*n* = 247), *ssa* (*n* = 170), *speJ* (*n* = 157), *speA* (*n* = 154), *speK* (*n* = 118), *speH* (*n* = 82), *speI* (*n* = 73), and *speL* and *speM*, which were always detected together (*n* = 44). The association of individual SAg genes with disease presentation was tested. In the analysis of these results, the SAg genes *speG* and *smeZ* were not considered because they were present in nearly all isolates, and the genes *speL* and *speM* were considered as a single entity, since they always co-occurred. Individually, the genes *speA* and *speJ* were both associated with invasive isolates ($P < 0.001$). As expected, strains carrying both these genes were also associated with invasive infections ($P < 0.001$), but no synergistic effect between the two genes was observed, since the presence of one did not significantly increase the representation of the other among invasive isolates. In contrast, *speC* ($P = 0.002$), *ssa* ($P < 0.001$), and *speL/M* ($P < 0.001$) were individually associated with pharyngitis. The combinations *speC+speL/M* and *ssa+speL/M* were both associated with pharyngitis ($P = 0.004$ and 0.012, respectively), but there was also no synergistic effect relative to the presence of a single gene. However, the association of *speC* with pharyngitis isolates can be explained by a high frequency of co-occurrence of this gene with *ssa*, since the isolates harboring *speC* without *ssa* were not significantly associated with any of the groups. An interesting situation occurred when analyzing the interaction between *speJ* (associated with invasive infections) and *ssa* (associated with pharyngitis). Among isolates carrying *speJ*, the group that also carried *ssa* was no longer associated with invasive infections, while the association of isolates carrying *ssa* with pharyngitis was not significantly altered by the presence of *speJ*. This argues for a dominant effect of the presence of *ssa* over that of *speJ* in determining the invasive capacity of individual isolates. The association of SAg profiles with disease presentation was also tested. Two SAg profiles presented a significant association with invasive isolates, namely SAg10 ($speA^+speG^+speJ^+smeZ^+$) and SAg46 ($speG^+smeZ^+$) ($P < 0.001$). The remaining profiles were not significantly associated with any of the two groups of isolates.

When the same kind of analysis was performed for *emm* types and individual SAg genes, three combinations with statistical significance emerged: the association of isolates presenting *emm*1 and *speA*, and *emm*1 and *speJ* with invasive infections ($P < 0.001$), and the association of isolates carrying *emm*75 and *speL/M* with pharyngitis ($P = 0.001$). In all cases,

no synergistic or antagonistic interaction was detected between *emm* type and SAg gene, since the *emm* type did not alter the association of the SAg gene with a particular group of isolates.

Differences between the PFGE clusters found among invasive infection and pharyngitis

The associations described above can be correlated with the PFGE clusters which were also different between the invasive and pharyngitis groups of isolates ($P < 0.001$), in agreement with the differences found in *emm* types (Figure III-1 and Figure III-2). All the 19 major PFGE clusters occurred in both invasive and pharyngitis isolates, except for R_6 (*emm*75-T25-ST150-SAg39), which was present only among pharyngeal isolates, but the difference did not reach statistical significance due to the small number of isolates in this cluster. PFGE distinguished several groups of isolates belonging to *emm* types 1 and 4. The difference in the distribution between pharyngitis and invasive infection was not found for all PFGE clusters containing isolates carrying each of these two *emm* types, but only for those including macrolide-susceptible isolates (B_{49} associated with invasive infections and F_{29} associated with pharyngitis, respectively, $P < 0.001$). The *emm*1 and *emm*4 isolates expressing macrolide resistance (M phenotype) were grouped into PFGE clusters O_9 and G_{27}, respectively, which presented a similar prevalence among invasive infections and pharyngitis (Figure III-2). PFGE J_{16}, which included all *emm*64 isolates, was also associated with invasive infections ($P < 0.001$). The *emm*75 association with pharyngitis was not translated into an association of a specific PFGE cluster, since the 19 *emm*75 strains were scattered into various PFGE clusters (Table III-2 and Table III-3).

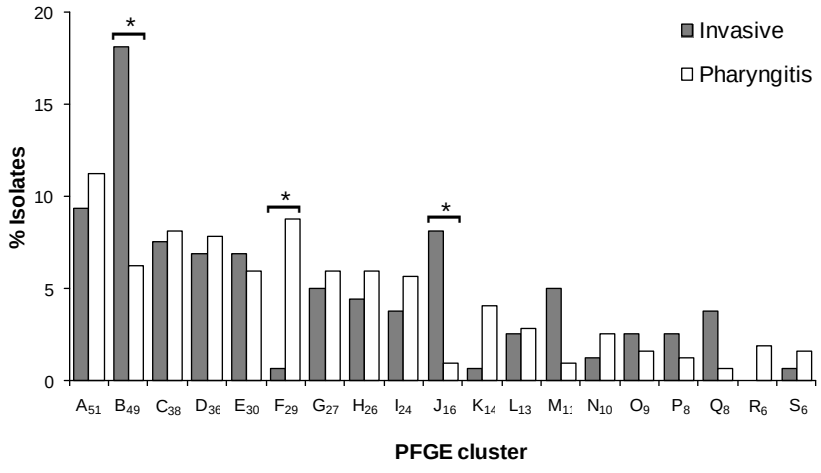

Figure III-2 PFGE clusters found among 160 invasive isolates and 320 pharyngitis isolates. Approximately 11% of invasive and 16% of non-invasive isolates were included in PFGE clusters of ≤ 5 isolates that are not represented. The asterisk indicates significant differences ($P<0.001$).

Not surprisingly, three *emm*-PFGE cluster combinations showed significant associations with infection type: *emm*1-B_{49} and *emm*64-J_{16} were associated with invasive infections, while *emm*4-F_{29} was associated with pharyngitis ($P < 0.001$). It was not possible to detect any synergistic or antagonistic interaction between PFGE and *emm* type in modulating the association of the isolates with either group. The same was true for the statistically significant combinations between PFGE clusters and individual SAg genes, namely the combination of B_{49} with *speA* and with *speJ* (both associated with invasive infections, $P < 0.001$), and the combination of F_{29} with *speC* and with *ssa* (both associated with pharyngitis, $P < 0.001$).

DISCUSSION

Several studies yielding conflicting results have attempted to compare the clonal composition of GAS populations causing invasive and non-invasive infections in order to identify particularly virulent clones or properties that may be used as epidemiological markers of invasiveness [12, 22, 27, 33, 34]. However, many of those studies were limited in the size and diversity of the GAS collections studied or in the typing methodologies used, with most of them relying essentially on *emm* typing, which has been shown to be insufficient for the complete identification of GAS clones [5]. In this work, we used several different typing methods to compare a collection of genetically diverse GAS isolates recovered from normally sterile sites during a period of six years in Portugal [18] with isolates recovered from pharyngeal exudates of patients presenting with tonsillo-pharyngitis, during the same time period and in the same geographical region. The nasopharyngeal mucosa has been suggested to be the main reservoir for GAS isolates associated with invasive infections [6, 15].

The differences among the GAS clones associated with invasive infections and pharyngitis were reflected in antimicrobial resistance, with the invasive group of isolates presenting lower macrolide resistance and higher tetracycline resistance, when compared with the pharyngitis group. Among isolates belonging to the same *emm* types, namely *emm*1 and *emm*4, only the macrolide-susceptible clones were associated with either invasive infections or pharyngitis. The macrolide-resistant clones of these *emm* types are reflected in invasive infections according to their prevalence in pharyngitis, suggesting that these are translating more the antibiotic selective pressure than the invasive capacity of the clones. Tetracycline is not currently used in the treatment of GAS infections but resistance to this antimicrobial in *S. pyogenes* is usually acquired by horizontal transfer, since the resistance genes are frequently encoded in mobile genetic elements with a wide host range [1]. These elements often carry macrolide resistance genes as well, and in *S. pyogenes* a significant association between the presence of the genes *erm*(B) and *tet*(M) has been reported and it has been suggested that tetracycline use could contribute to the selection of macrolide-resistant GAS isolates [1, 28]. In our study, the association between the presence of the genes *erm*(B) and *tet*(M) was observed globally, but not among the invasive isolates, suggesting that the genetic elements carrying tetracycline resistance conferring genes may be different between the two bacterial populations.

Bacitracin susceptibility is routinely used for the presumptive identification of GAS, although resistant clones have been identified in several countries [24, 26, 43]. In our GAS collection, all the bacitracin-resistant isolates (5%), regardless of the type of infection, were clustered in the same PFGE clone (H_{26}) and belonged to ST52, although one was *emm*22-T12 while the others were all *emm*28-T28. Isolates with such characteristics had been previously reported in Portugal associated with tonsillo-pharyngitis, skin infections and asymptomatic carriage [31, 37], but not with invasive infections. Bacitracin resistance among invasive isolates has been previously reported only among isolates recovered in France and in San Francisco [26, 43].

Although 74% of the invasive isolates in our collection belonged to clones which were equally frequent among pharyngitis, suggesting that a significant part of the invasive GAS population mirrors the clonal structure of the circulating GAS isolates, the remaining isolates represented clones that had an enhanced capacity to cause invasive disease. We also found significant associations between individual properties or pairwise combinations of properties and disease presentation. Since in most cases these were also characteristics of the more invasive clones, we cannot exclude that the associations of individual properties or pairwise combinations of properties can reflect, at least partially, the distribution of genetic lineages in the two GAS populations analyzed.

Individually, *emm* types 1 and 64 were associated with invasive infections. Isolates belonging to these *emm* types presented the only two SAg profiles significantly associated with invasive infections. Surprisingly, only one of these SAg profiles includes a phage-encoded SAg gene (*speA*). In agreement with our observation, a previous study found that within the same PFGE-*emm* group, the SAg profiles significantly associated with invasive infections had a smaller number of SAg genes than the dominant profiles in pharyngitis [9]. These results suggest that although some SAg genes may significantly contribute to the virulence of *S. pyogenes*, the rise and success of highly virulent GAS clones may not hinge upon the acquisition of phage-encoded SAg genes. Still, in our study, the SAg genes *speA* and *speJ* were both significantly more prevalent among invasive isolates. This association was not substantially affected by *emm* type, PFGE clone, nor by the presence of other SAg genes, suggesting that *speA* and *speJ* can be regarded by themselves as markers for invasiveness. Although such association has not been previously noted for *speJ*, the *speA* gene has been frequently associated with invasive infections [7, 22, 27] and the production of SpeA by GAS isolates has been linked to streptococcal toxic shock syndrome [32].

On the other hand, we identified an association of pharyngitis isolates with *emm* types 4 and 75, and with the SAg genes *speC*, *ssa*, and *speL*/*M*. The association of *speC* with non-invasive infections has been previously reported [7, 19, 22], but in our collection this association could be explained simply by a high frequency of co-occurrence of this gene with *ssa* which was strongly associated with pharyngitis, as was also noted in a recent study [22]. The presence of the genes *speL* and *speM* was not previously associated with non-invasive infections.

Since there is a strong correlation of the SAg profile with *emm* type and of both these properties with PFGE type, some of these individual factors frequently co-occurred in the same clones. Therefore, combinations of these characteristics were also significantly associated with disease presentation. However, we could not detect any synergistic or antagonistic interactions between most of these characteristics, meaning that their co-occurrence in a particular isolate does not make it more invasive than isolates sharing only one of these characteristics.

Two PFGE clusters were significantly more prevalent among isolates associated with invasive disease than among those causing tonsillo-pharyngitis. One of these was a cluster of macrolide-susceptible isolates characterized as *emm*1-T1-ST28 and by the presence of the SAg genes *speA*, *speG*, *speJ*, and *smeZ* (B_{49}), which accounted for 18% of the invasive isolates. M1T1 isolates have been frequently associated with severe invasive GAS disease, and the acquisition of prophage-encoded virulence genes, as well as horizontal gene transfer events by homologous recombination were implicated in the increased virulence of these isolates [2, 39]. However, many epidemiological studies found a similar prevalence of this clone among invasive and non-invasive isolates [10, 33, 34], questioning its enhanced invasive capacity. In contrast to these findings, but similarly to those of others [7, 12, 27, 42], we found an association between this clone and invasive GAS disease in Portugal, although it can also frequently cause milder infections such as pharyngitis (it accounted for 6% of the pharyngitis isolates analyzed in this study).

The other cluster significantly associated with invasive infections in Portugal was J_{16}, which was dominated by isolates belonging to *emm*64-ST164 and carrying the SAg genes *speG* and *smeZ*. A clone with these characteristics has not been previously associated with invasive disease and *emm*64 has been infrequently reported among invasive GAS isolates [23, 29, 40]. The higher invasive capacity of this clone cannot be attributed to its SAg repertoire, since these isolates do not harbor any of the SAg genes associated with invasive infection. Other, still unidentified, characteristics may be responsible for the properties of this clone.

In contrast to these PFGE clones, the F_{29} clone of macrolide-susceptible isolates characterized by *emm*4-T4-ST39 and harboring the genes *speC*, *ssa* and *smeZ* was associated with pharyngitis, suggesting that this clone may have a reduced ability to cause invasive disease, in agreement with the negative association between *emm*4 and invasive infection that has been suggested elsewhere [22]. The association of *emm*75 with pharyngitis has not been previously reported and was not translated into particular PFGE clusters due to the high diversity of *emm*75 isolates.

Our data confirms that the widely dispersed M1T1 clone has enhanced invasiveness but we also identified clones with increased or decreased invasive capacity that may have emerged locally and that have a more limited temporal and geographical spread. The *emm* alleles and the SAg genes characteristic of these clones were associated with particular disease presentations. Other individual *emm* alleles and SAg genes were also associated with a higher propensity to cause invasive infections or pharyngitis indicating the importance of these characteristics in determining an isolate's invasive capacity.

Other factors that were not evaluated in this study may contribute to a different distribution of GAS clones in less severe and more severe infections. These include bacterial factors, such as the occurrence of mutations in transcriptional regulators controlling the expression of virulence factors, which seems to play an important role in the pathogenesis of some GAS isolates [20]. For other clones, the ability to cause invasive infections may be more dependent on exploiting host factors, like the HLA class II haplotype [21], which may vary in frequency in different human populations.

CONCLUSIONS

This study established links between particular genetic lineages and the type of infection, indicating that genetic characteristics of the bacteria play an important role in determining the outcome of their interaction with the human host. The different distribution of clones in the two types of infection supports the relevance of PFGE as a typing methodology for GAS [5]. This was further evidenced by the fact that the macrolide-resistant *emm*1 and *emm*4 PFGE clones were not associated with any particular disease presentation, contrary to the susceptible clones carrying the same *emm* types that were associated with invasive infections and pharyngitis, respectively. Moreover, in contrast to other reports [34, 35] we found associations between particular *emm* alleles and SAg genes and disease presentation. In this study, we identified *emm*4, *emm*75, *ssa* and *speL/M* as independent markers for pharyngitis and *emm*1, *emm*64, *speA*, and *speJ* as independent markers for invasiveness. Our data re-enforces the multi-factorial nature of GAS invasive capacity and highlighted lineages and characteristics, in addition to the well known M1T1 lineage, that are associated with particular disease presentations and that may further increase in importance.

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SUPPLEMENTARY DATA

Table III-S SAg genes profiles identified in GAS isolates in Portugal.

SAg profile	No. of isolates	*speA*	*speC*	*speG*	*speH*	*speI*	*speJ*	*speK*	*speL*	*speM*	*ssa*	*smeZ*
1	1	+	+	-	-	-	-	-	-	-	+	+
2	31	+	+	+	-	-	-	+	-	-	-	+
3	2	+	+	+	-	-	+	-	-	-	-	+
4	2	+	+	+	-	-	-	-	+	+	-	+
5	2	+	+	+	-	-	-	-	-	-	-	+
6	1	+	-	+	+	+	+	-	-	-	+	+
7	1	+	-	+	+	-	+	-	-	-	-	+
8	49	+	-	+	-	-	-	+	-	-	+	+
9	1	+	-	+	-	-	-	+	-	-	-	+
10	57	+	-	+	-	-	+	-	-	-	-	+
11	7	+	-	+	-	-	-	-	-	-	-	+
12	6	-	+	+	+	+	+	-	-	-	+	+
13	3	-	+	+	+	+	-	-	-	-	+	+
14	1	-	+	+	+	-	-	-	-	-	+	+
15	2	-	+	+	+	+	+	+	-	-	-	+
16	15	-	+	+	+	+	-	-	-	-	-	+
17	3	-	+	+	+	-	-	-	-	-	-	+
18	1	-	+	+	-	-	+	+	-	-	+	+
19	3	-	+	+	-	-	-	+	-	-	+	+
20	5	-	+	+	-	-	+	-	-	-	+	+
21	7	-	+	+	-	-	-	-	-	-	+	+
22	3	-	+	-	-	-	-	-	+	+	+	+
23	52	-	+	-	-	-	-	-	-	-	+	+
24	17	-	+	+	-	-	+	+	-	-	-	+
25	2	-	+	+	-	-	-	+	+	+	-	+
26	2	-	+	+	-	-	-	+	-	-	-	+
27	44	-	+	+	-	-	+	-	-	-	-	+
28	3	-	+	+	-	-	-	-	+	+	-	+
29	21	-	+	+	-	-	-	-	-	-	-	+
30	3	-	+	-	-	-	-	-	-	-	-	+
31	16	-	+	+	-	-	-	-	+	+	-	-
32	16	-	-	+	+	+	+	-	-	-	+	+
33	30	-	-	+	+	+	-	-	-	-	-	+
34	1	-	-	+	+	-	+	-	-	-	-	+
35	3	-	-	+	+	-	-	-	-	-	-	+
36	1	-	-	+	-	-	-	+	+	+	+	+
37	2	-	-	+	-	-	-	+	-	-	+	+
38	2	-	-	+	-	-	+	-	-	-	+	+
39	8	-	-	+	-	-	-	-	+	+	+	+
40	8	-	-	+	-	-	-	-	-	-	+	+
41	1	-	-	-	-	-	-	-	-	-	+	+
42	2	-	-	+	-	-	-	+	+	+	-	+
43	5	-	-	+	-	-	-	+	-	-	-	+
44	2	-	-	+	-	-	+	-	-	-	-	+
45	5	-	-	+	-	-	-	-	+	+	-	+
46	26	-	-	+	-	-	-	-	-	-	-	+
47	2	-	-	-	-	-	-	-	-	-	-	+
48	2	-	-	+	-	-	-	-	+	+	-	-
49	1	-	-	-	-	-	-	-	-	-	-	-

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CHAPTER IV

SUPERANTIGEN GENE COMPLEMENT OF *STREPTOCOCCUS PYOGENES* – RELATIONSHIP WITH OTHER TYPING METHODS AND SHORT-TERM STABILITY

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SUMMARY

The profiling of the superantigen (SAg) encoding genes has been frequently used as a complementary typing method for group A streptococci (GAS), but a confusing gene nomenclature and a large diversity of primers used in screening has led to some conflicting results. The aim of this work was to develop a PCR method capable of efficiently amplifying all the known allelic variants of these genes, and to evaluate the congruence of this methodology with other commonly used molecular typing methods. The presence of the 11 known SAg genes and two other exotoxin-encoding genes (*speB* and *speF*) was tested in a collection of 480 clinical GAS isolates, using two multiplex PCR reactions. The SAg gene profile was compared with other typing methods. Four naturally occurring deletions involving the genes *speB*, *speF*, and *rgg* were characterized, two of which were found among invasive isolates. The absence of the chromosomally encoded genes *speG* and *smeZ* was supported by Southern blot hybridization and associated with specific GAS lineages, while the presence of phage-encoded genes was more variable. Positive associations between SAg genes or between SAg profiles and *emm* types or pulsed-field gel electrophoresis (PFGE) clusters were observed. The results suggest that the SAg profile diversifies faster than other properties commonly used for molecular typing, such as *emm* type and multilocus sequence typing (MLST) sequence types, and can be a useful complement in GAS molecular epidemiology. Still, the short-term stability of the SAg gene profile among prevalent genetic lineages may largely explain the observed associations between SAg genes.

INTRODUCTION

Streptococcus pyogenes (mostly – to simplify matters – also referred to as Group A streptococcus, GAS) can cause a variety of human diseases, ranging from uncomplicated superficial infections of the respiratory tract and skin to severe invasive disease associated with high morbidity and mortality [12]. Among the major virulence factors of GAS are the secreted pyrogenic exotoxins (SPE), which act as superantigens (SAg) due to their ability to interact with the host MHC class II molecules and with the variable region of the T cell receptor β-chain without previous processing by antigen-presenting cells. This interaction results in the activation of a large number of T cells expressing specific V_β subsets without activation by specific antigens, leading to the hypotension and multiorgan failure observed in streptococcal toxic shock syndrome (STSS) [24, 41]. To date, 11 distinct SAgs have been identified in GAS: 3 chromosomally encoded (SpeG, SpeJ, and SMEZ) and 8 encoded on temperate phages (SpeA, SpeC, SpeH, SpeI, SpeK, SpeL, SpeM, and SSA). SpeB and SpeF, both encoded on the bacterial chromosome, were originally described as SPEs, but in fact they are not true SAgs but a cysteine protease and a DNase, respectively [18, 23]. Multiple allelic forms have been recognized in *speA*, *speC*, *speG*, *ssa*, and *smeZ* that may be associated with variations in superantigenic activity and in antigenic properties [7, 41].

Identification of GAS clones in surveillance and epidemiological studies relied frequently on serotyping using two variable surface antigens – the T antigen (T typing) and the M protein (M typing) [12]. However, these serological methods are limited to the diversity of typing sera available and are being replaced by molecular methods. In recent years, sequencing of the hypervariable region of the *emm* gene, which encodes the M protein (*emm* typing) [4], has become the gold standard of GAS molecular typing for surveillance and epidemiological purposes, although there is evidence that it should be complemented with other typing methods, like pulsed-field gel electrophoresis (PFGE) macrorestriction profiling or multilocus sequence typing (MLST), in order to fully discriminate GAS clones [9]. The determination of the exotoxin gene profile has also been commonly used as a method for molecular typing, since it may provide means to further discriminate GAS isolates analysed by *emm* typing [26, 29, 31]. There is evidence that the exchange and integration of mobile genetic elements, such as prophages, among GAS isolates is a strong contributor to the genomic diversification of this species and to the emergence of highly successful virulent clones [6, 30, 43]. Distinct studies present significant differences regarding the prevalence of

SAg genes that are considered as chromosomally encoded, as well as the association between exotoxin gene profile and *emm* type. The majority of recent studies rely on PCR for screening of the SAg genes. The use of primers that do not cover all allelic variants or that anneal nonspecifically with the genome, as well as the existence of non-functional remnants of some of these genes, may lead to the occurrence of false negative or false positive PCR results and these may explain, at least in part, the conflicting results reported in the literature.

In this work, we developed a method to screen GAS isolates for the presence of the 11 SAg genes, using the amplification of the chromosomally encoded genes *speB* and *speF* as control for successful PCR reactions. The assay involves two multiplex PCR reactions with internal primers designed according to the 13 GAS genomes published at the time and to the known allelic variants of the genes. The *speK* gene in our study refers to the *speK* identified in the genome sequence of strain MGAS315 (M3) [6] and to the gene described as *speL* in prophage PhiNIH1.1 [20]. On the other hand, *speL* and *speM* are the genes originally described in the M18 isolate MGAS8232 (AE009949) [40]. The two genes assigned as *speL* and *speM* by Proft *et al*. [34] correspond to *speK* and *speL*, respectively, in the present study. The genes *speM* and *speK* are highly similar in the 5' and 3' ends, a fact that led to the use of the same forward primer to amplify both genes. The screening was performed in a collection of 480 GAS isolates recovered from both pharyngitis and invasive infections in Portugal that were also typed by other molecular methods, in order to evaluate the congruence of the SAg gene profile with other typing methods. Negative PCR results for *speB* and *speF* were confirmed by sequencing and four distinct *in vivo* genetic deletions covering these important virulence factors were characterized.

MATERIALS AND METHODS

Bacterial Isolates

A total of 480 non-duplicate GAS isolates collected from human infections in 32 laboratories distributed throughout Portugal (2000-2005) were studied. The invasive isolates (*n* = 160) were collected from normally sterile sites, and their partial characterization was previously reported [16]. A total of 320 isolates submitted by the same laboratories were randomly selected from 1604 isolates recovered from pharyngeal exudates of patients presenting with tonsillo-pharyngitis.

Phenotypic and molecular typing

Typing of GAS isolates was performed by T typing, *emm* typing, PFGE, and MLST as reported elsewhere [16, 38, 39].

SAg genes profiling

Template DNA was obtained through the incubation of bacteria with mutanolysin and hyaluronidase, according to the protocols and recommendations of the CDC (Centers for Disease Control and Prevention, http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). The presence of the 11 SAg genes was tested in two multiplex PCR reactions using specific primer pairs designed to cover all the known allelic variants of each gene. The primers were designed inside the genes, based on the 13 GAS genome sequences published at the time (GenBank AE004092, AE009949, AE014074, AM295007, BA000034, CP000003, CP000017, CP000056, CP000259-CP000262, CP000829) and all *speA*, *speC*, and *smeZ* sequences available in GenBank. The amplification fragments of genes *speB* and *speF* were used as controls for successful PCR reactions. Table IV-1 presents all primer sequences used, as well as the expected fragment sizes. Reaction 1 was used to screen for the presence of *speF*, *speG*, *speH*, *speJ*, *speK*, *ssa*, and *smeZ* and was carried out in a total volume of 25 µl, containing 1× Green GoTaq Flexi Buffer (Promega, Madison, WI, USA), 5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer (except for ssa1/ssa2 and speJg-f2/speJg-r – 10 pmol each), 2 U of GoTaq DNA Polymerase (Promega), and 1 µl of template DNA. The remaining genes (*speA*, *speB*, *speC*, *speI*, *speL*, and *speM*) were screened in reaction 2, which was performed in a total volume of 25 µl containing 1× Green GoTaq Flexi Buffer (Promega), 6 mM $MgCl_2$,

0.2 mM each dNTP, 20 pmol of primers speK-f, speM-r, speL-f, speL-r, speI1, and speI3, 10 pmol of primers speC-f2, speC-r2, speB1, speB2, speA-f2, and speA-r2, 1.5 U of GoTaq Flexi DNA Polymerase (Promega), and 1 µl of template DNA. The PCR cycle for both reactions was as follows: initial denaturing for 2 min at 95ºC, followed by 40 cycles of 1 min at 95ºC, 30 s at 54ºC (reaction 1) or 62ºC (reaction 2), and 1 min 30 s at 72ºC. PCR products were separated and visualized in ethidium-bromide stained 1% (reaction 1) or 2% (reaction 2) agarose gels. DNA of strain SF370, which contains the genes *speB*, *speF*, *speG*, *smeZ*, *speC*, *speH*, *speI*, and *speJ*, was mixed with DNA of selected isolates containing the remaining genes to be used as positive control in each batch of multiplex PCR reactions.

Table IV-1 Primers used for exotoxin gene profiling by multiplex PCR.

Gene	Primers (5'→3')	Size of PCR product (bp)	Reference
Exotoxin gene profiling			
speA	speA-f2 – AAGAACC(A/T/G)AGAGATG(T/G)CAAC speA-r2 – ATAG(G/A)CTTTGGATACCATC(G/A)	202	[22], adapted
speB	speB1 – TTCTAGGATACTCTACCAGC speB2 – ATTTGAGCAGTTGCAGTAGC	300	[22]
speC	speC-f2 – CAGTCATACTGATTTCTACTATTTCACC speC-r2 – CAAGATAAATATCGAAATGACTAAAGTTC	660	This study
speF	speF1 – CGAAATTAGAAAAGAGGAC speF2 – GGCTGAGCAAAAGTGTGTG	1193	[10]
speG	speG-f – GTATCTTTAGGCATTACTGATC speG-r – GTTTACTATCTTTAGTAGCAAG	340	This study
speH	speH1 – TTCAAGCAAATTCTTATAATACAACC speH3 – AAAGTCTCCATTGCCAAAATAATAC	545	This study
speI	speI1 – TATGAGATGAGTAGTGTGGGAGTTATTAA speI3 – GTTCCTGAATCGTAACCTCTTTCTTATC	411/412	This study
speJ	speJg-f2 – CGAGAGCTATATTACAACAAAG speJg-r – CACTCCTTGTACTAGATGAGG	386	This study
speK	speK-f – TATCGCTTGCTCTATACACTACTGAGAGT speK-r2 – CTTATCTTTAGCTGTTAATGTTCGTAATTC	478/479	[25] This study
speL	speL-f – GGACGCAAGTTATTATGGATGCTCA speL-r – TTAAATAAGTCAGCACCTTCCTCTTTCTC	460	[25]
speM	speK-f (see above) speM-r – ATGAGTGAATAAATCGGTAAACTTTGTTG	587	[25] This study
ssa	ssa1 – GTGTAGAATTGAGGTAATTG ssa2 – TAATATAGCCTGTCTCGTAC	706	[36]
smeZ	smeZ-F – TAGAAGTAGATAATAATTCC smeZ-R2 – TTAGGAGT(C/T)AATTTCTATAT	628/629/616	[35], adapted
Characterization of *speB*/*speF* deletions			
	sp-up2 - GCTTTATGACATTGCCCTAGACCATCAG	N/A[a]	This study
	sp-up3 - GTCAACACTCCAGCACTCAACAAGGC	N/A[a]	This study
	sp-dn2 - GACTATCTCTTCTTATGGTTAAGGATACG	N/A[a]	This study

[a] Different combinations of the primers sp-up2, sp-up3, sp-dn2 and speF1 were used resulting in fragments of different sizes, as explained in the text.

Southern blot hybridization

The absence of the chromosomal genes *speB*, *speF*, *speG*, *speJ*, and *smeZ*, determined by PCR, was confirmed by Southern blot hybridization. Since *speG*, *speJ*, and *smeZ* have been previously reported as absent in some GAS lineages [11, 29, 35, 36, 42], Southern blot hybridization was performed for two representative isolates of each major PFGE cluster (defined as a group of > 5 isolates with a Dice coefficient similarity ≥ 80% in the UPGMA dendrogram) presenting strains that lacked each or a combination of the genes *speG*, *speJ* and *smeZ*. Whenever possible, isolates of the same PFGE clone but belonging to different *emm* types or MLST sequence types (STs) were selected to maximize the genetic diversity of the studied isolates. The probes were prepared by single PCR using primers speB1/speB2, speF1/speF2, speG-f/speG-r, speJg-f2/speJg-r, or smeZ-f/smeZ-r2, and DNA of SF370 or a suitable isolate from our collection.

Characterization of chromosomal deletions

Genomic DNA of the isolates lacking *speB* and/or *speF*, as well as of strain SF370 (used as control due to the availability of its complete genome sequence) was extracted using CTAB [3]. Long-range PCR with primers external to the deleted genes was performed with the Expand Long Template PCR System (Roche, Mannheim, Germany) according to the manufacturer's instructions. The PCR products were then sequenced by primer walking and the resulting sequences were compared with the published genome sequence of strains SF370 (GenBank AE004092), MGAS6180 (GenBank CP000056), or MGAS10750 (GenBank CP000262) using Vector NTI software (Invitrogen, Paisley, UK).

Statistical analysis

The results of the typing methodologies were analyzed and compared using the Simpson's index of diversity (SID) and the Adjusted Wallace coefficient (AW) and corresponding 95% confidence intervals ($CI_{95\%}$) [9, 32, 37]. Individual associations between SAg genes, SAg profiles, *emm* type and PFGE type were screened by computing Jaccard coefficients [21], odd-ratios and Fisher exact tests [1]. In each screen, the subset of positive or negative associations was defined according to odd-ratio value, and the corresponding subset of p-values of the Fisher exact test were corrected for multiple testing through the False Discovery Rate (FDR) linear procedure [5]. The p-value threshold to consider a significant

association was such that the FDR was equal or lower than 0.05. Jaccard coefficient values were used to represent the strength of significant associations. When a variable divides strains in numerous mutually exclusive groups, like SAg profiles, *emm* and PFGE types, it is only meaningful to analyze positive associations. Small groups increase the number of negative associations just by chance, confounding their interpretation. In the case of associations between individual SAg genes, both positive and negative associations were analyzed, although FDR correction was done separately, due to the dependence between both types of associations (for example, if two SAg genes tend to co-occur, it is expected to observe the same set of negative associations for both SAg genes).

RESULTS

SAg gene profile determination

The two multiplex PCR reactions allowed the detection of 13 gene fragments by conventional agarose electrophoresis (Figure IV-1). The *speF* gene was absent in only one isolate (0.2%), which was also negative for *speB*. The latter was absent in a total of four isolates (0.8%).

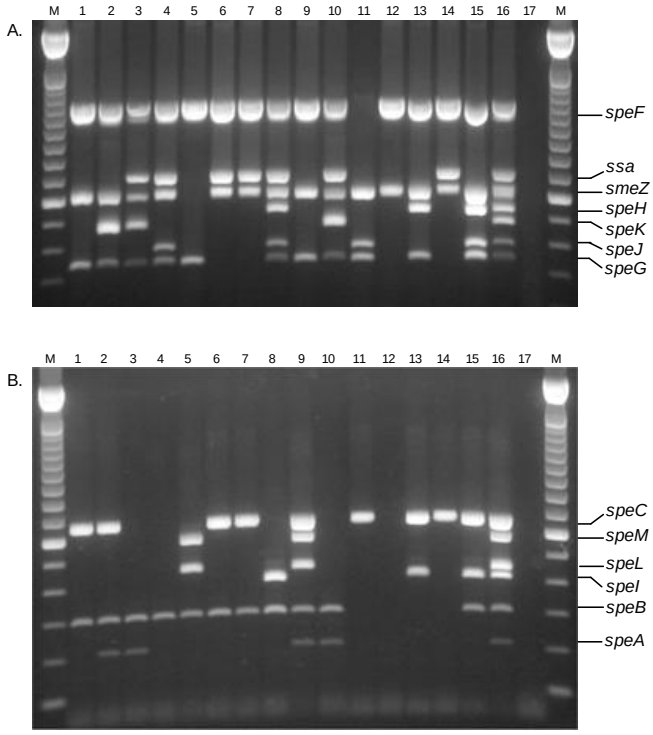

Figure IV-1 SAg genes profiles obtained by agarose gel electrophoresis of multiplex PCR reactions. A. – multiplex PCR reaction 1; B. – multiplex PCR reaction 2; M – 100 bp DNA ladder marker (Invitrogen, Paisley, UK). 1-15 – representative GAS isolates with different SAg profiles; 12-15 – isolates without the genes *speB* and/or *speF*; 16 – Positive control DNA, containing all the gene fragments expected in each multiplex reaction; 17 – negative control, without DNA.

The chromosomally located SAg genes *smeZ*, *speG*, and *speJ* were present in 461 (96.0%), 417 (86.9%), and 157 (32.7%) isolates, respectively. The absence of these genes in two isolates of each PFGE cluster was further supported by Southern blot results (data not shown). Among the phage encoded genes, *speC* was the most prevalent (*n* = 247, 51.5%), followed by *ssa* (*n* = 170, 35.4%), *speA* (*n* = 154, 32.1%), *speK* (*n* = 118, 24.6%), *speH* (*n* = 82, 17.1%), *speI* (*n* = 73, 15.2%), and *speL* and *speM*, which were always detected together (*n* = 44, 9.2%).

Forty-nine distinct SAg gene profiles were identified (Table IV-2) and none of them was present in more than 12% of the isolates, showing high diversity (Table IV-3).

Table IV-2 Superantigen (SAg) genes profiles identified in group A streptococci (GAS) isolates in Portugal.

SAg profile	No. of isolates	*speA*	*speC*	*speG*	*speH*	*speI*	*speJ*	*speK*	*speL*	*speM*	*ssa*	*smeZ*	*emm* types[a] (No. of isolates)
1	1	+	+	-	-	-	-	-	-	-	+	+	4
2	31	+	+	+	-	-	-	+	-	-	-	+	3(2), **6 (28)**, 87 (1)
3	2	+	+	+	-	-	+	-	-	-	-	+	1(1), 22 (1)
4	2	+	+	+	-	-	-	-	+	+	-	+	**18**
5	2	+	+	+	-	-	-	-	-	-	-	+	6 (1), **74 (1)**
6	1	+	-	+	+	+	+	-	-	-	+	+	89
7	1	+	-	+	+	-	+	-	-	-	-	+	75
8	49	+	-	+	-	-	-	+	-	-	+	+	**3 (48)**, 22 (1)
9	1	+	-	+	-	-	-	+	-	-	-	+	6
10	57	+	-	+	-	-	+	-	-	-	-	+	**1(56)**, stIL103 (1)
11	7	+	-	+	-	-	-	-	-	-	-	+	**18 (2), 43 (4), 74 (1)**
12	6	-	+	+	+	+	+	-	-	-	+	+	**44**
13	3	-	+	+	+	+	-	-	-	-	+	+	**22**
14	1	-	+	+	+	-	-	-	-	-	+	+	**58**
15	2	-	+	+	+	+	+	+	-	-	-	+	**28**
16	15	-	+	+	+	+	-	-	-	-	-	+	**11(4)**, **12 (11)**
17	3	-	+	+	+	-	-	-	-	-	-	+	**58**
18	1	-	+	+	-	-	+	+	-	-	+	+	**87**
19	3	-	+	+	-	-	-	+	-	-	+	+	**22**
20	5	-	+	+	-	-	+	-	-	-	+	+	**87**
21	7	-	+	+	-	-	-	-	-	-	+	+	**22**
22	3	-	+	-	-	-	-	-	+	+	+	+	**4**
23	52	-	+	-	-	-	-	-	-	-	+	+	**4**
24	17	-	+	+	-	-	+	+	-	-	-	+	**28**
25	2	-	+	+	-	-	-	+	+	+	-	+	**75**
26	2	-	+	+	-	-	-	+	-	-	-	+	**53**
27	44	-	+	+	-	-	+	-	-	-	-	+	**28 (16)**, 77 (1), 87 (3), **89 (21)**, **102 (3)**
28	3	-	+	+	-	-	-	-	+	+	-	+	**75**
29	21	-	+	+	-	-	-	-	-	-	-	+	11 (1), 25 (1), 30 (1), 48 (1), **78 (8)**, **89 (8)**, stG1750 (1)
30	3	-	+	-	-	-	-	-	-	-	-	+	4 (2), **77 (1)**
31	16	-	+	+	-	-	-	-	+	+	-	-	**2**
32	16	-	-	+	+	+	+	-	-	-	+	+	**44**
33	30	-	-	+	+	+	-	-	-	-	-	+	**12**
34	1	-	-	+	+	-	+	-	-	-	-	+	**103**
35	3	-	-	+	+	-	-	-	-	-	-	+	**94**
36	1	-	-	+	-	-	-	+	+	+	+	+	75
37	2	-	-	+	-	-	-	+	-	-	+	+	**3**
38	2	-	-	+	-	-	+	-	-	-	+	+	44 (1), 87 (1)
39	8	-	-	+	-	-	-	-	+	+	+	+	**75**
40	8	-	-	+	-	-	-	-	-	-	+	+	4 (1), **9 (6)**, 89(1)
41	1	-	-	-	-	-	-	-	-	-	+	+	4
42	2	-	-	+	-	-	-	+	+	+	-	+	**75**
43	5	-	-	+	-	-	-	+	-	-	-	+	58 (1), 64 (1), **89 (3)**
44	2	-	-	+	-	-	+	-	-	-	-	+	87 (1), **118 (1)**
45	5	-	-	+	-	-	-	-	+	+	-	+	**29 (2)**, **75 (2)**, **90 (1)**
46	26	-	-	+	-	-	-	-	-	-	-	+	9 (2), 11 (2), 12 (1), 53 (1), **64 (10)**, **89 (6)**, **113 (3)**, 114 (1)
47	2	-	-	-	-	-	-	-	-	-	-	+	**77**
48	2	-	-	+	-	-	-	-	+	+	-	-	**2**
49	1	-	-	-	-	-	-	-	-	-	-	-	**st106M**

[a] *emm* types significantly associated with each SAg profile are presented in **bold**, according to Figure IV-S2.

Table IV-3 Simpson's index of diversity (SID) and 95% confidence intervals ($CI_{95\%}$) for the several typing methods used in the analysis of the 480 GAS isolates.

Typing method	No. of partitions	SID [$CI_{95\%}$]
emm typing	36	0.928 [0.921-0.935]
T typing	17	0.916 [0.910-0.922]
PFGE80[a]	46	0.946 [0.940-0.953]
PFGE95[b]	178	0.980 [0.975-0.985]
Superantigen (SAg) profiling	49	0.938 [0.931-0.945]
Chromosomal SAg profiling	5	0.622 [0.595-0.649]
Phage SAg profiling	32	0.916 [0.908-0.925]
emm+T typing	70	0.945 [0.938-0.952]
emm+SAg profiling	84	0.949 [0.942-0.957]
MLST[c]	49	0.960 [0.950-0.970]

[a] Groups defined at 80% similarity on a Dice/UPGMA dendrogram. These are frequently referred to as PFGE types.
[b] Groups defined at 95% similarity on a Dice/UPGMA dendrogram. These are frequently referred to as PFGE subtypes.
[c] The SID for MLST was calculated among a subset of 170 isolates for which the MLST analysis was performed. The MLST diversity is not comparable to that of other methods because MLST was performed only in a subset of isolates that were not randomly chosen. Within each PFGE80 major cluster (grouping > 5 isolates), isolates belonging preferentially to different *emm* types and T types were selected for MLST analysis, introducing a diversity bias in the MLST sample.

Characterization of chromosomal deletions involving *speB* and *speF*

The *speB* and *speF* genes are encoded in the same region and are separated by approximately 2 kb including the gene encoding the transcriptional regulator RopB/Rgg (Figure IV-2).

The isolates 2002V1512P (*emm*77-T13-ST550-SAg47, isolated from blood), 2004V1141P (*emm*11-T11-ST562-SAg16, isolated from pharyngeal exudate), and 2005V0398P (*emm*4-T4-ST39-SAg23, isolated from pharyngeal exudate) lacked the *speB* gene, although *speF* was present. The isolate 2000V1032P (*emm*28-T28-ST52-SAg27, isolated from blood), was negative for both the *speB* and *speF* genes. Sequencing of this region showed relatively small deletions in two isolates: 1095 bp (2002V1512P) encompassing part of *speB* and most of CDS897, and of 967 bp (2004V1141P) encompassing most of the *speB* gene and 50 bp of its 5' flanking region.

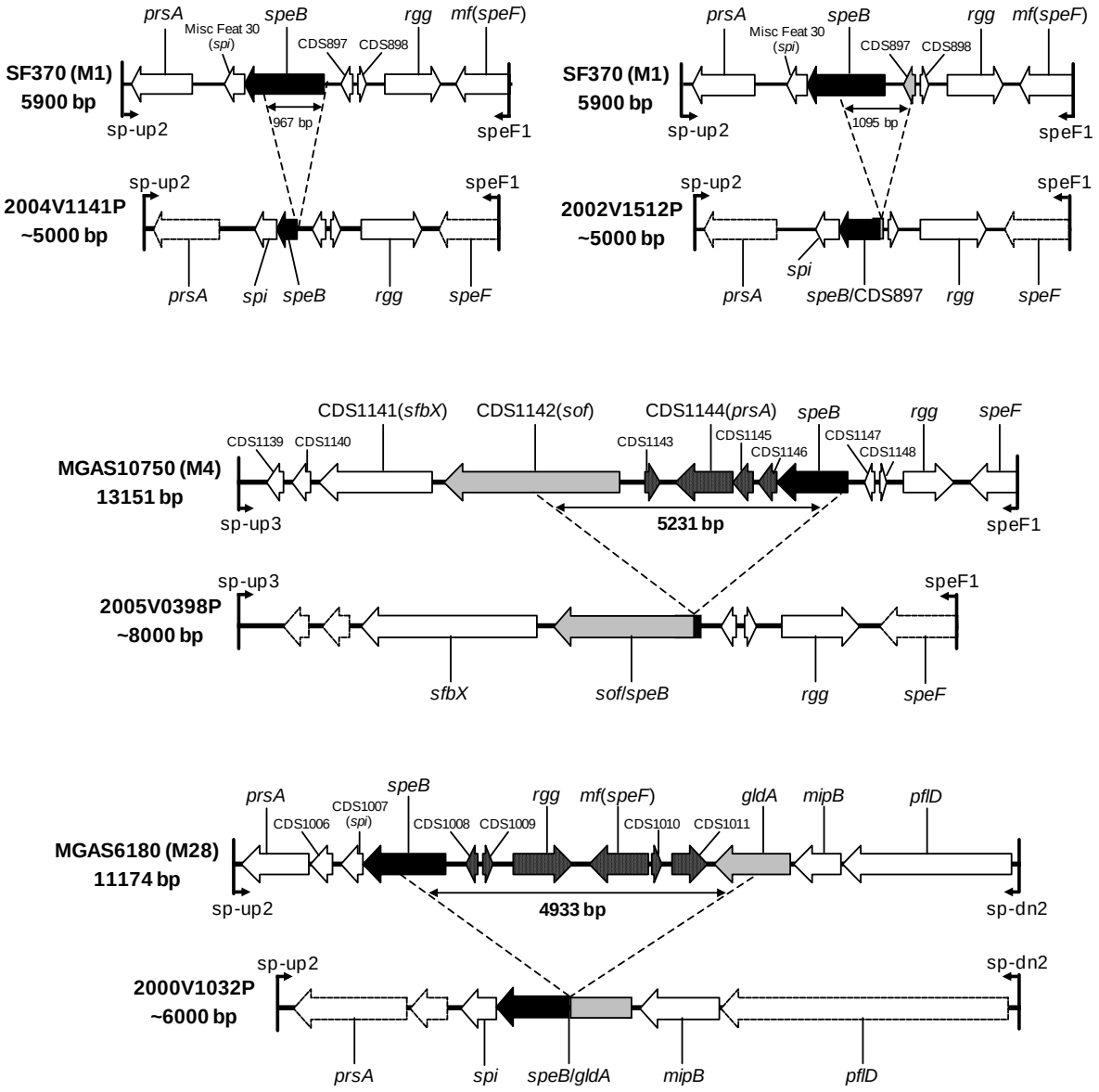

Figure IV-2 The four types of chromosomal deletions identified in the locus encoding the genes *speB* and *speF*. Genes are indicated by arrows: black and gray arrows for genes containing the deletion breakpoints, crosshatched arrows for completely deleted genes, white solid line arrows for genes not involved in the deletion, and white broken line arrows for genes not completely sequenced in our isolates. Primers used for long-range PCR are represented by vertical lines with arrow ends. For each of the four isolates characterized, the approximate size of the fragment obtained by long-range PCR is indicated next to the name of the strain. Published genomes of GAS strains of the same *emm* type were used for comparison, except for 2004V1141P (*emm*11) and 20021512P (*emm*77), for which the comparison was performed with the genome of strain SF370, due to the unavailability of genome sequences of isolates with the same *emm* types.

In isolate 2005V0398P a larger 5231 bp deletion was identified encompassing nearly half of CDS1142 (*sof* gene, encoding the serum opacity factor), the complete ORFs CDS1143, CDS1144 (*prsA* gene, encoding a peptidylpropyl isomerase), CDS1145, CDS1146, and almost the complete *speB* gene.

In 2000V1032P a 4933 bp deletion was identified encompassing part of *speB*, *rgg*, *mf* (*speF*), part of *gldA* (encoding glycerol dehydrogenase), and four ORFs encoding putative

proteins (CDS1008-1012) (Figure IV-2). In contrast to the previous deletions the remaining portions of *speB* and *gldA* originate a new ORF that could result in a fusion protein.
The sequences obtained for each of the four isolates carrying deletions were deposited in GenBank, with nucleotide accession numbers JF724128-JF724131.

SAg gene associations

The associations between the SAg genes were analysed by measuring their pairwise co-occurrence through the Jaccard coefficient. This coefficient will be 1 if the two SAg genes are always present in the same strains, and 0 if they are never present simultaneously in one strain. The positive associations with statistical significance are represented in Figure IV-3. Despite being the most frequent phage-encoded gene, *speC* was not significantly associated with any of the other genes. The *speL* and *speM* genes were only associated with each other, possibly as a consequence of their low frequency among our collection that reduced the statistical power to detect associations with other genes. All remaining genes presented at least two significant positive associations. The strongest associations were between *speL* and *speM* (Jaccard = 1), *speI* and *speH* (Jaccard = 0.890), and *speK* and *speA* (Jaccard = 0.424).

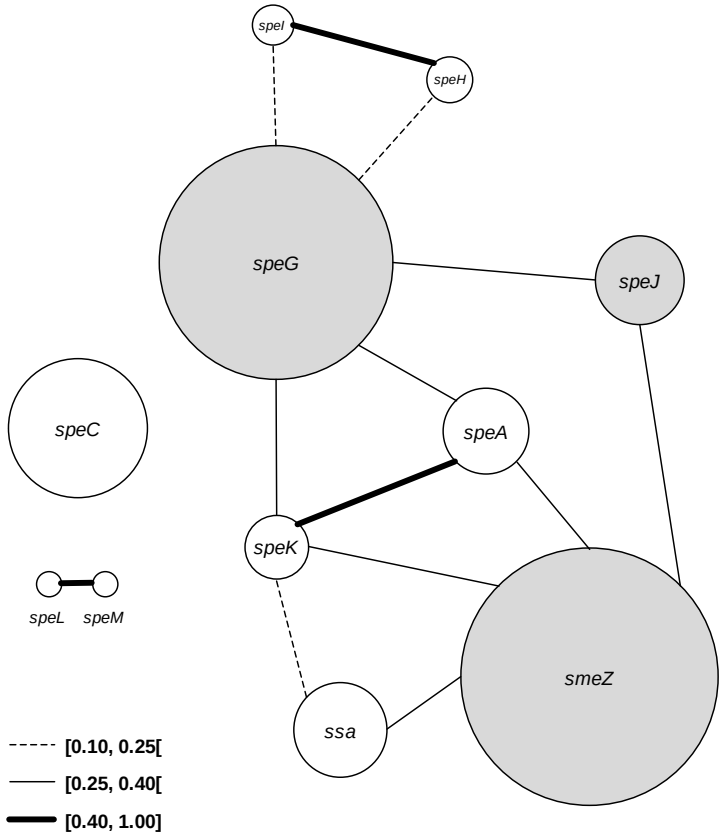

Figure IV-3 Representation of the statistically significant positive associations between the 11 SAg genes. Each gene is represented by a circle of size that is proportional to the frequency of that gene among the 480 GAS isolates. Chromosomal genes are represented in gray. The lines correspond to Jaccard coefficients > 0.10 according to the legend.

Comparison of typing methods

All typing methods presented high levels of diversity (SID > 0.9) with the exception of the chromosomal SAg profile (Table IV-3). The complete SAg profile presented a higher SID than the phage-encoded SAg profile alone, indicating that the chromosomally encoded SAg genes contribute significantly to the diversity of the SAg repertoire.

The association between the different typing methods was evaluated using the AW coefficients presented in Table IV-S. Individually, all SAg genes presented significant associations with several emm types and PFGE clusters (Figure IV-S1). A similar situation was found for the complete toxin profile (Figure IV-S2). The association between PFGE80 and *emm* (Figure IV-S3) largely justifies the parallels shown in the panels of Figures IV-S1 and IV-S2.

DISCUSSION

The exotoxins with superantigenic properties produced by GAS play an important role in the pathogenesis of this species due to their ability to induce an exacerbated, non-specific inflammatory response, which is at the origin of some of the most severe symptoms associated with serious GAS infections. Therefore, the profiling of the genes encoding such toxins has been frequently used to evaluate the capacity of a given strain causing more severe infections as well as in molecular typing of *S. pyogenes* isolates [26, 29, 31]. However, published data show very contrasting results concerning the frequency of some SAgs, including those believed to be chromosomally encoded. We explored these differences by analyzing an extensive and genetically diverse collection of GAS isolates using primers suitable for amplifying all the known allelic variants of the SAgs, and by further supporting the deletions found in chromosomal genes with Southern blot results.

The *speB* and *speF* genes do not encode true SAgs, but were used in this work as positive PCR control fragments, since they are reported to be present in virtually all GAS strains. Despite their chromosomal origin, several studies have reported the absence of these genes in a low frequency of GAS isolates [13, 31, 36, 42] relying on PCR techniques and the potential deletions involving the genes were not characterized. In this work, we performed the first unambiguous characterization of naturally occurring deletions involving *speB* and *speF*. Four distinct chromosomal deletions were detected. We found no clonal expansion of isolates harboring them, consistent with such deletions not being advantageous to the bacteria. However, two of the isolates presenting deletions in the *speB-speF* region, including the isolate with a large deletion encompassing *speB*, *rgg*, and *speF*, were recovered from blood and were therefore associated with systemic infections. Both the cysteine protease SpeB and the transcriptional regulator Rgg/RopB have been implicated in GAS pathogenesis, although their role as virulence enhancers or repressors has not been clearly defined [2, 19, 28]. The occurrence of deletions involving *speB* and *rgg* in invasive isolates suggests a non-essential role of these genes for the establishment of GAS invasive disease, at least in some isolates. However, detailed clinical information regarding the patients from which these isolates were obtained would be needed to further substantiate this hypothesis. On the other hand, the actual impact of SpeB and Rgg in virulence may vary in different GAS lineages.

The existence of several allelic variants in some SAg genes has possibly resulted in reporting of false-negatives due to the use of primers that are not able to amplify all alleles.

On the other hand, some of the SAg genes were initially described at approximately the same time by different authors in different strains, resulting in the assignment of distinct names to the same gene [33]. Therefore, it has become increasingly difficult to accurately compare the SAg gene profiling results from different studies. In this work, all primers used were checked against all the SAg gene sequences present in GenBank, in order to ensure that all the allelic variants described at the time could be efficiently amplified by the two multiplex PCR reactions developed.

As expected, the chromosomally encoded genes *smeZ* and *speG* were present in the great majority of isolates (96% and 87%, respectively), but both were notably absent in a limited number of *emm* types, as evidenced in this study by both PCR and Southern hybridization. The *smeZ* gene was only absent in isolates belonging to four PFGE clusters, all presenting with either *emm* type 2 or *emm* type *st*106M. The use of primers located in polymorphic regions of this gene, which has 50 allelic variants deposited in GenBank, may have artificially inflated the number of isolates previously identified as lacking *smeZ* [25, 36, 42]. Isolates without *speG* were distributed into nine PFGE clusters but presented only *emm*4, *emm*77, and *st*106M. These data indicate that both *smeZ* and *speG* are absent from a limited number of GAS lineages, supporting their chromosomal origin, and suggesting that the contrasting results found in studies reporting their absence in large numbers of lineages are probably caused by the methodology used [25, 36, 42]. However, it is not possible to exclude that in some geographical regions other clones also lacking these genes may be found.

In contrast, *speJ*, which is also considered a chromosomal gene, is reported in this and other studies as absent in GAS isolates from diverse lineages [11, 31, 35, 36]. The *speJ* gene is suggested to have been acquired from a temperate phage that was subsequently lost from the genome of descendants of this original GAS lineage [30] but multiple independent acquisitions of this phage cannot also be excluded.

Concerning the phage-encoded SAg genes, in our study *speC* was the most prevalent, being detected in more than half of the isolates, followed by *ssa* and only then by *speA*. These results are in agreement with the great majority of epidemiological GAS studies [11, 27, 29]. The genes *speL* and *speM* were detected in a small fraction of the isolates, but were always found together in the same strains, which is in accordance with their contiguous position in the same prophage [40]. In contrast, *speI* and *speH*, despite being also in tandem in a prophage [15], were independently detected in nine isolates in our study (all harboring *speH*, but not *speI*) belonging to different lineages. This supports the hypothesis that *speI* is

sometimes lost during phage integration [11], in contrast to a more stable association between *speL* and *speM*.

Several positive associations between SAg genes were detected among our collection of GAS isolates, in contrast to a previous study that found positive associations only for pairs of genes encoded on the same prophage, namely *speL-speM* and *speH-speI* [11]. As expected, the strongest associations were also found for those genes, but many other positive correlations were identified and the link between *speK* and *speA* was particularly strong. Such associations have not been previously reported and are not explained by the co-localization of the genes in the same mobile genetic elements. Perhaps not surprisingly, most of the associations involve the genes of chromosomal origin *speG* and *smeZ*. The *speK* gene, in spite of its phage origin, has four positive correlations, two with the chromosomal *speG* and *smeZ*, and another two with genes encoded in different phages, namely *speA* and *ssa*. The *speK* gene was also significantly associated with PFGE80 clusters 10, 13 and 17 (Figure IV-S1). PFGE cluster 13 (*emm*6) was significantly associated with *speA*, and PFGE cluster 17 (*emm*3) was positively linked to both *speA* and *ssa*. These clonal relationships, in addition to the prevalence of both PFGE cluster 13 (6.3%) and 17 (10.6%) among the studied isolates, help explain the strong association observed between *speK* and *speA*, as well as the weaker but significant link between *speK* and *ssa*. The co-occurrence of *speK*, *speA*, and *ssa* in the M3-sequenced strain MGAS315 (presenting ST15, which was also found among our *emm*3 clone [17]) has been attributed to a sequential acquisition of different prophages by an ancestral M3 strain [6]. Our PFGE cluster included ST15 and its single-locus variants, suggesting ongoing differentiation of this original lineage.

Epidemiological typing of GAS has relied on a variety of typing methodologies, from the long used T- and M-serotyping, to the more recent sequence-based techniques like *emm* typing and MLST. The profiling of the SAg gene content of GAS isolates has also been presented as a useful complementary typing method [26, 29, 31]. The higher diversity and predictive capacity over other typing methods of the 11 SAg profile when compared with the 8 phage-encoded SAg profile supports the usefulness of determining the complete profile of SAg genes (Table IV-3). The SID for the 11 SAgs profiling in our collection was similar to the one obtained in a recent study for the screening of 20 GAS virulence factors (including the 11 SAgs) using four multiplex PCR reactions [8]. This suggests that the profiling of the remaining virulence factors probably does not bring relevant additional information when compared with the SAg profiling alone, and a simpler protocol of only two PCR reactions, as

proposed herein, can be used. The multiplex PCR reactions presented in our study have the additional advantage of including positive control fragments (*speB* and *speF*).

In the collection of GAS isolates analysed, none of the individual SAg genes was linked to a specific *emm* type since all presented significant associations with at least three different *emm* types. However, we did confirm a strong association between the complete SAg profile and *emm* type reported previously [11, 36, 42], with most of the profiles of the 11 genes being significantly associated with one *emm* type (Figure IV-S2). The converse was also true and although the majority of the *emm* types included isolates of more than one SAg profile, most of them were significantly linked to one or two profiles at most (Figure IV-S2). Still, based on the respective AW coefficients, we concluded that the SAg profile was a better predictor of *emm* type than the reverse. The SAg profile could not be confidently predicted by any of the other typing methods except the PFGE subtype, which groups isolates with at least 95% similarity in the dendrogram, as previously proposed for *Staphylococcus aureus* [14]. This observation is in agreement with a recent study [8] in which the authors concluded that differences in virulence factor profiles are reflected by subgroups of PFGE patterns, in a highly clonal collection of strains. Our study extends this conclusion for a genetically diverse collection of GAS isolates. PFGE subtyping discriminates the isolates into a very high number of partitions and is able to translate a much shorter time scale of genetic diversification. This suggests that the SAg profile varies in the population at a faster rate than the most commonly used GAS typing methods, like *emm* and MLST, reflecting the exchange of SAg genes by lateral genetic transfer events. Therefore, the data presented herein supports the usefulness of SAg profiling as a complementary typing method for GAS that may help to further discriminate and identify GAS clones presenting specific properties, such as enhanced virulence or association with specific infections. Moreover, the SAg genes can also be considered markers for the presence and transfer of prophages that may encode additional genes playing a role in GAS pathogenesis or tissue tropism.

Discrepancies found in the literature concerning the frequency of the several SAg genes may be essentially explained by (i) methodology issues associated with primer design and gene nomenclature and (ii) a different clonal composition of the GAS populations analysed in the several studies. The two multiplex PCR reactions proposed provide a rapid and simple means for determining the complete SAg gene profile of large collections of GAS strains. The characterization of the SAg gene profile of extended GAS populations may afford new insights into the dynamics of transmission of these important virulence factors and may help explain the emergence and diversification of particularly virulent strains.

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All the experimental work regarding the determination of the SAg gene content of the isolates, the characterization of the deletions involving *speB* and *speF*, and the molecular typing of the invasive isolates was performed by A. Friães. The molecular typing (except SAg gene profiling) of the isolates associated with pharyngitis was performed by A. Friães (50%) and C. Silva-Costa (50%). Statistical analysis was performed by F. R. Pinto.

SUPPLEMENTARY DATA

Table IV-S Adjusted Wallace values (95% confidence intervals) for the several typing methods used in the analysis of the 480 GAS isolates.

Typing method	*emm* typing	T typing	PFGE80	PFGE95	SAg profiling	*emm*+T typing	*emm*+SAg typing	MLST (ST) [a]
***emm* typing**		0.736 (0.688-0.783)	0.680 (0.637-0.723)	0.252 (0.210-0.295)	0.684 (0.635-0.733)	0.744 (0.698-0.790)	0.688 (0.640-0.736)	0.692 (0.619-0.766)
T typing	0.622 (0.575-0.669)		0.421 (0.376-0.467)	0.153 (0.120-0.187)	0.427 (0.379-0.475)	0.629 (0.583-0.675)	0.420 (0.371-0.468)	0.291 (0.225-0.357)
PFGE80	0.932 (0.904-0.961)	0.683 (0.639-0.727)		0.355 (0.304-0.406)	0.705 (0.657-0.753)	0.678 (0.633-0.723)	0.700 (0.650-0.749)	
PFGE95	0.974 (0.937-1.000)	0.700 (0.638-0.762)			0.953 (0.933-0.973)	0.686 (0.618-0.754)	0.935 (0.895-0.975)	
SAg profiling	0.803 (0.774-0.832)	0.593 (0.546-0.639)	0.603 (0.561-0.646)	0.290 (0.242-0.338)		0.586 (0.539-0.633)	0.807 (0.779-0.836)	0.563 (0.496-0.631)
***emm*+T typing**			0.665 (0.619-0.711)	0.239 (0.195-0.283)	0.671 (0.620-0.722)		0.675 (0.625-0.726)	0.811 (0.756-0.865)
***emm*+SAg typing**		0.722 (0.669-0.774)	0.742 (0.700-0.785)	0.352 (0.297-0.407)		0.730 (0.680-0.781)		0.756 (0.678-0.834)
MLST (ST) [a]	0.964 (0.919-1.000)	0.673 (0.575-0.772)			0.650 (0.589-0.711)	0.670 (0.577-0.764)	0.629 (0.556-0.703)	

[a] Values involving MLST were calculated for a subset of 170 isolates for which the MLST analysis was performed. The association with PFGE was not analysed, due to the bias introduced in this sample, as explained in Table IV-3.

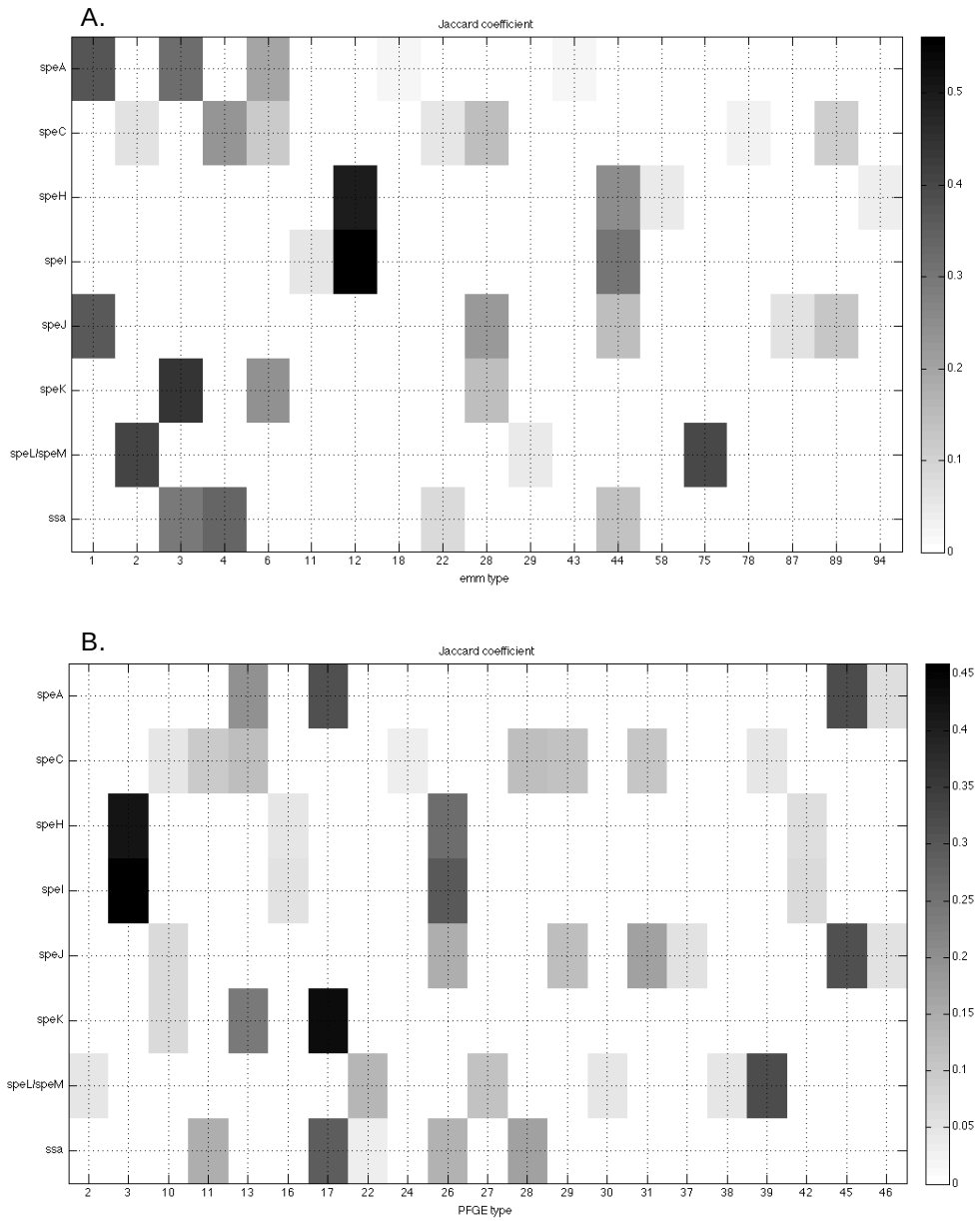

Figure IV-S1 Relationship between individual SAgs and *emm* and PFGE. The significant Jaccard coefficients obtained when comparing all SAgs with *emm* types (panel A) and PFGE80 clusters (panel B) detected are represented. Lighter shades of gray represent weaker associations, while darker shades of gray represent stronger associations.

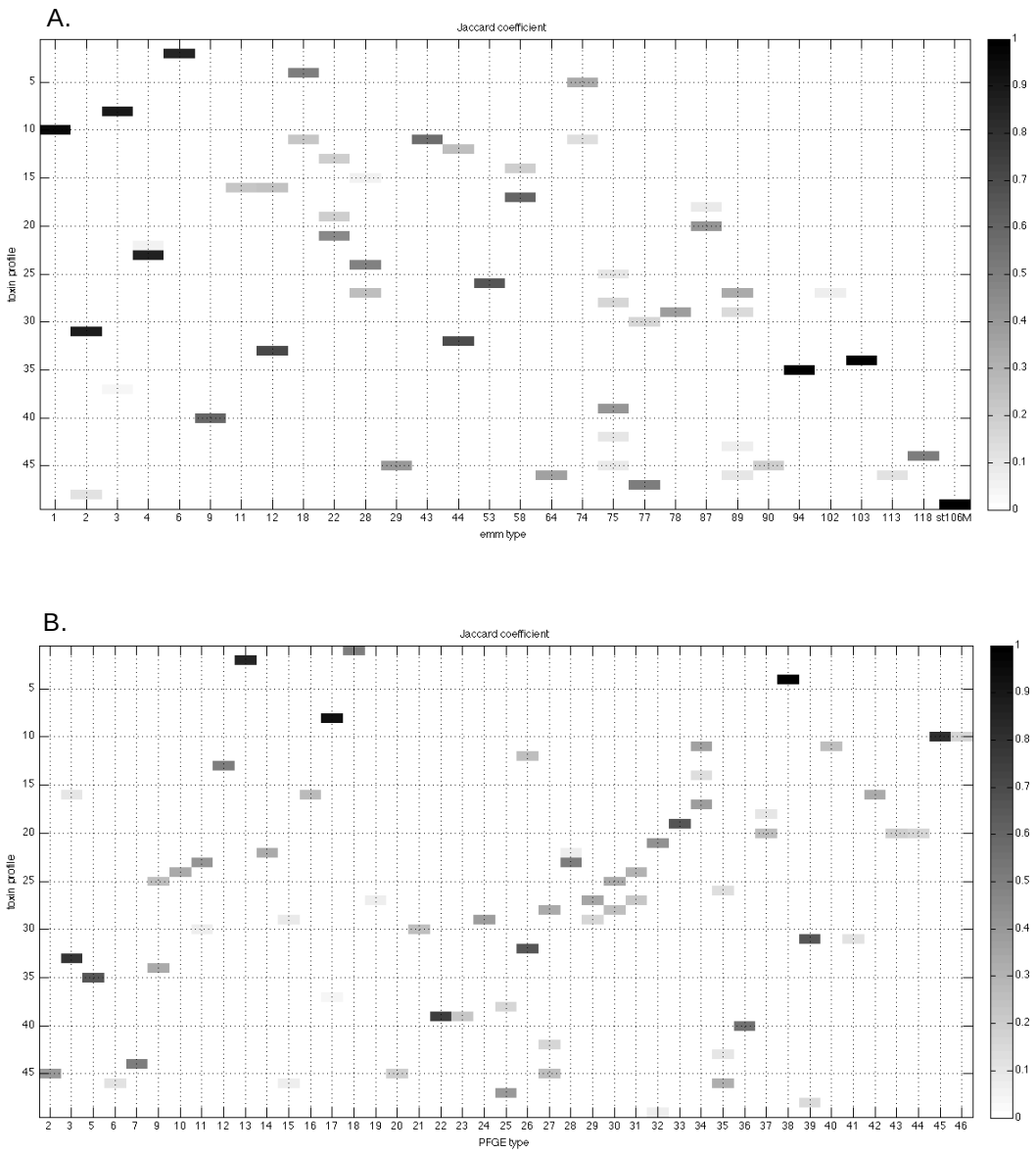

Figure IV-S2 Relationship between SAg profile and *emm* and PFGE. The significant Jaccard coefficients obtained when comparing all SAg profiles with *emm* types (panel A) and PFGE80 clusters (panel B) detected are represented. Lighter shades of gray represent weaker associations, while darker shades of gray represent stronger associations.

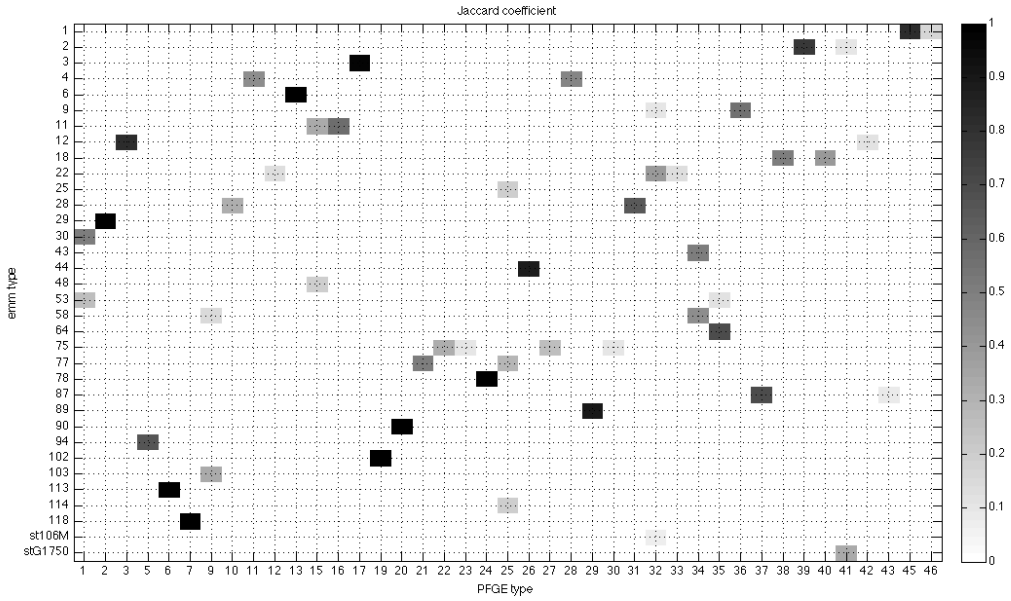

Figure IV-S3 Relationship between *emm* and PFGE. The significant Jaccard coefficients obtained when comparing all *emm* types detected with all PFGE80 clusters are represented. Lighter shades of gray represent weaker associations, while darker shades of gray represent stronger associations.

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CHAPTER V

CHANGES IN *STREPTOCOCCUS PYOGENES* CAUSING INVASIVE DISEASE IN PORTUGAL: EVIDENCE FOR SUPERANTIGEN GENE LOSS AND ACQUISITION

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SUMMARY

The emergence of highly virulent and successful *Streptococcus pyogenes* (group A streptococci – GAS) clones has been attributed to the exchange of virulence factors by lateral gene transfer mechanisms, which strongly contribute to genomic diversity. We characterized a collection of 191 GAS isolates recovered from normally sterile sites in Portugal during 2006-2009 and compared them to invasive isolates obtained during 2000-2005. Antimicrobial resistance rates did not change significantly between the two periods and were generally low. In 2006-2009, *emm*1, *emm*89, *emm*3, and *emm*6 represented 60% of the isolates. The chromosomally encoded superantigen (SAg) genes *speG* and *smeZ* were present in the majority (>90%) of the isolates, while *speJ* was found in only 45%. The phage encoded SAgs varied greatly in prevalence (2-53%). The distribution of *emm* types, pulsed-field gel electrophoresis profiling (PFGE) clusters, and SAg profiles changed significantly between the periods, although there were no statistically supported changes in the prevalence of individual types. While the macrolide susceptible clone *emm*1-T1-ST28 remained dominant (28%), there was a significant decrease in clonal diversity as indicated by both PFGE profiling and *emm* typing. This was accompanied by intra-clonal divergence of SAg profiles, which was statistically confirmed for isolates representing *emm*1, *emm*28, and *emm*44. This diversification was associated with the loss and acquisition of SAg genes, carried by phages and of chromosomal origin. These data suggest an ongoing genomic diversification of GAS invasive isolates in Portugal that may contribute to the persistence of clones with improved fitness or virulence.

INTRODUCTION

Streptococcus pyogenes (Group A Streptococci, GAS) is able to cause a wide variety of human infections, ranging from uncomplicated tonsillo-pharyngitis or impetigo, to severe and life threatening invasive infections, including necrotizing fasciitis, bacteremia, streptococcal toxic shock syndrome (STSS), pneumonia, puerperal sepsis, and meningitis [8]. An increase in the incidence of severe GAS infections has been documented since the late 1980's in several developed countries, both in North America and in Europe [10]. Recent studies indicate that during the first decade of 2000, invasive infections maintained a high incidence in Europe and remained associated with high mortality and morbidity [21, 23, 41].

Despite *S. pyogenes* remaining universally susceptible to penicillin, the drug of choice for the treatment of uncomplicated GAS infections, its efficacy in monotherapy for the treatment of complicated GAS infections has been questioned. In these cases, the association of penicillin with clindamycin has been advocated [46], but resistance to the latter has been described in GAS. This is frequently associated with resistance to macrolides in isolates expressing the MLS_B phenotype. Moreover, macrolide resistance determinants are often carried by mobile genetic elements which also harbor tetracycline resistance genes [1]. Levofloxacin is not a therapeutic option for severe GAS infections, but the emergence of fluoroquinolone non-susceptible GAS clones, mostly belonging to *emm*6, *emm*11, and *emm*75, has been reported in several countries, including among invasive isolates [26, 30, 37]. Intermediate fluoroquinolone resistance is usually caused by first-step mutations in the quinolone resistance-determining region (QRDR) of the *parC* gene, while high level resistance usually requires a second-step mutation, most frequently in the QRDR of the *gyrA* gene, and is much less frequent among GAS isolates [26, 30, 37].

Currently, the most widely used molecular typing method for the epidemiological surveillance of GAS clones is based on the sequencing of the hypervariable region of the *emm* gene encoding the M protein [2]. However, molecular epidemiological data suggests that *emm* typing must be complemented with other molecular typing methods, like pulsed-field gel electrophoresis macrorestriction profiling (PFGE) or multilocus sequence typing (MLST) in order to fully discriminate GAS clones [4]. Profiling the set of superantigen (SAg) genes carried by each isolate is also commonly used as a typing methodology that may help to further discriminate the GAS clones defined by other typing methods [16, 24]. Eleven SAg genes have been described so far: *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, and *ssa* are

encoded on bacteriophages, while *speG*, *speJ*, and *smeZ* are usually considered to be encoded on the bacterial core genome, although *speJ* has been lost from most GAS lineages [16, 27].

The streptococcal exotoxins encoded by the SAg genes are recognized as important virulence factors due to their ability to induce an unspecific, exacerbated inflammatory response in the host [44]. Although the exact role of each of these exotoxins in the pathogenesis of invasive infections is still controversial, the lateral transfer of mobile genetic elements, like prophages, among GAS isolates has been considered one of the main mechanisms contributing to diversification in this species and to the emergence of highly successful and virulent clones [27, 47, 50]. The overall increase in the pathogenicity of *emm*1 and *emm*28 isolates was proposed to have been originated by a sequential acquisition, during the decades of 1950 and 1960, of phages carrying SAg genes like *speA2*, *speC*, and *speK*, along with genes encoding other virulence factors like the DNases SdaD2 or Sdn and a phospholipase [50]. Hence, the acquisition of SAg genes may directly contribute to an increase in GAS virulence, since the production of some SAgs has been previously associated with the occurrence of STSS [31, 38], or it may be regarded as a marker for the acquisition of sets of virulence factors during the same horizontal gene transfer events. On the other hand, the presence of additional genes not contributing to GAS pathogenesis or fitness may represent an unnecessary metabolic burden to the bacterium, in which case the loss of those genes may bring an evolutionary advantage. In support of the latter hypothesis, some studies report a low number of phage-encoded SAg genes in the clones significantly associated with invasive infections [9, 18].

Studies from Europe report significant changes in the clonal composition of invasive GAS isolates during recent years. The *emm*1-T1-ST28 remains the dominant clone, although it presented a decreasing trend in Germany and France, while increasing in Finland [19, 25, 36, 41]. Contrasting shifts in the prevalence of *emm* types 28 and 89 were also reported [7, 19, 29, 36, 41].

In a previous study, we characterized GAS isolates associated with invasive infections, collected throughout Portugal during 2000-2005 and found a genetically diverse population, dominated by a macrolide susceptible clone characterized by *emm*1-T1-ST28 and carrying the genes *speA*, *speG*, *speJ*, and *smeZ* [17, 18]. In Portugal, as well as in other countries, this clone was found to be overrepresented among isolates recovered from normally sterile sites, when compared with isolates associated with tonsillo-pharyngitis [6, 11, 18, 29]. The same observation was made for a clone defined by *emm*64-ST164, which does not carry any of the phage-encoded SAg genes [18].

In the present work, we characterized a collection of GAS isolates obtained from normally sterile sites throughout Portugal, during 2006-2009 (*n* = 191), using *emm* typing, T typing, PFGE macrorestriction profiling, SAg gene profiling, and MLST. All isolates were also tested for susceptibility to clinically and epidemiologically relevant antimicrobial agents. The results were compared to those previously obtained for the collection of 2000-2005, in order to identify possible shifts in the clonal composition of the GAS population causing invasive infections in Portugal during the last decade, as well as to identify possible changes in the SAg gene content of the main circulating clones.

MATERIALS AND METHODS

Bacterial Isolates

A total of 191 non-duplicate GAS isolates were recovered from normally sterile fluids (173 from blood, 9 from pleural fluid, 3 from cerebrospinal fluid, 3 from ascitic fluid, and 3 from synovial fluid) in 25 laboratories distributed throughout Portugal, between 2006 and 2009 (38 isolates in 2006, 55 in 2007, 53 in 2008, and 45 in 2009). Strains were identified by the submitting laboratories and confirmed in our laboratory by colony morphology, β-hemolysis and the presence of the characteristic group antigen (Slidex Strepto A, BioMérieux, Marcy l'Etoile, France). Patient age ranged from nine months to 96 years with a median age of 54 years (38 patients were < 18 years, 88 were 18-64 years, and 65 were ≥ 65 years).

Antimicrobial susceptibility testing

Susceptibility tests were performed by disk diffusion on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, according to the guidelines and interpretative criteria of the Clinical and Laboratory Standards Institute (CLSI) [5], using the following antibiotic disks (Oxoid, Basingstoke, UK): penicillin, vancomycin, erythromycin, tetracycline, levofloxacin, chloramphenicol, clindamycin, quinupristin/dalfopristin, and linezolid. Intermediate susceptibility results were confirmed by MIC determination using E-test strips (BioMérieux, Marcy l'Etoile, France) and CLSI interpretative criteria [5]. The macrolide resistance phenotype was determined as previously described [28]. Susceptibility to bacitracin was determined for all isolates using disks containing 0.05 U of bacitracin (Oxoid, Basingstoke, UK), as described elsewhere [43].

Detection of genetic determinants of antimicrobial resistance

Bacterial DNA was prepared according to the protocols of the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Determination of the macrolide resistance genotype was performed for strains presenting either the M or the MLS_B macrolide resistance phenotype, by a multiplex PCR reaction with primers to detect the *erm*(B), *erm*(A) and *mef* genes [13]. Isolates carrying the *mef* gene were subjected to a second PCR reaction, using primers MEF1, MEF2, and MEFR as described elsewhere [42], in order to discriminate between *mef*(A) and *mef*(E). Tetracycline resistant

isolates were screened by PCR for the presence of the genes *tet*(K), *tet*(L), *tet*(M), and *tet*(O) as previously described [49]. Control strains harboring each of the resistance genes were used as positive controls for the PCR reactions. For the isolates presenting reduced levofloxacin susceptibility, the segments of the *gyrA* and *parC* genes containing the quinolone resistance-determining regions (QRDRs) were PCR-amplified and sequenced using previously proposed primers [20]. The obtained sequences were compared with the *gyrA* and *parC* sequences of a susceptible strain deposited in GenBank (accession numbers AF220945 and AF220946, respectively).

T typing

Strains were cultured in Todd-Hewitt broth (Oxoid, Basingstoke, UK) at 30ºC overnight and treated with swine pancreatic extract, using the Auxiliary Reagents for Hemolytic Streptococcus Typing (Denka Seiken, Tokyo, Japan), and following the manufacturer's instructions. T serotypes were determined by slide agglutination with 5 polyvalent and 19 monovalent sera (Hemolytic Streptococcus Group-A Typing Sera, Denka Seiken).

PFGE macrorestriction profiling

Agarose plugs of bacterial DNA were prepared as previously described [43]. After digestion with SmaI or Cfr9I (Fermentas, Vilnius, Lithuania), the fragments were resolved by PFGE as described elsewhere [43]. The macrorestriction patterns generated were compared, together with those previously obtained for 160 invasive isolates collected in Portugal between 2000 and 2005 [17], using the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) to create UPGMA (unweighted pair group method with arithmetic mean) dendrograms. The Dice similarity coefficient was used, with optimization and position tolerance settings of 1.0 and 1.5, respectively. Major PFGE clusters were defined as groups of ≥ 5 isolates presenting profiles with ≥ 80% relatedness on the dendrogram [4].

***emm* typing, SAg gene profiling, and MLST**

The *emm*-typing of all isolates was performed according to the protocols and recommendations of the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm), and the first 240 bases of each sequence were compared to sequences deposited in the CDC *emm*-

database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). The presence of the SAg genes *speA*, *speC*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *smeZ*, and *ssa*, and of the chromosomally encoded exotoxin genes *speB* and *speF* (used as positive control fragments) was tested for all isolates using two multiplex PCR reactions, as previously described [16]. MLST analysis was performed as described elsewhere [12] for one third of the isolates of each *emm* type represented by at least three isolates, as well as for all isolates belonging to *emm* types that are rare or were only recently deposited in the CDC database. A total of 62 isolates were characterized by MLST. Allele and sequence type (ST) identification was performed using the *S. pyogenes* MLST database (http://spyogenes.mlst.net). Whenever new alleles were identified, chromatograms of both strands were submitted to the database curator for approval and an allele number was assigned. Clonal complexes were determined using the goeBURST algorithm implemented in PHYLOViZ [14, 15].

Statistical analysis

The results of the typing methodologies were analyzed and compared using the Simpson's index of diversity (SID) and the adjusted Wallace coefficient (AW) with corresponding 95% confidence intervals ($CI_{95\%}$) [4, 34, 40]. These measures were calculated using an online tool (http://www.comparingpartitions.info). The SID indicates the probability of two strains sampled randomly from a population belonging to two different types, varying between 0 and 1. The higher the SID value, the higher the discriminatory ability of the typing method and the diversity of the population regarding the characteristic analyzed by that typing method. The AW coefficient represents the probability of a pair of strains that are assigned to the same type by one method being also classified in the same type by the other method corrected for chance agreement. A higher AW value (varying between 0 and 1) translates a higher ability of one typing method to predict the results of another. Differences between the collection of isolates analyzed in this study and the previously studied invasive isolates from 2000-2005 [17] were evaluated using the χ^2 or Fisher's exact test, correcting *P* values for multiple testing through the False Discovery Rate (FDR) linear procedure [3]. The FDR method will correct the *P* values of individual tests to take into account the fact that when performing multiple tests some are expected to fall below the *P* value of significance simply by chance. This is therefore a more conservative approach to significance than simply taking the individual *P* values. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Antimicrobial resistance

All isolates were fully susceptible to penicillin, quinupristin-dalfopristin, chloramphenicol, vancomycin, and linezolid.

A total of 16 isolates from 2006-2009 presented erythromycin resistance, resulting in a resistance rate (8%) that although lower, was not significantly different from the one reported for the period of 2000-2005 (12%) [18]. Thirteen of these isolates were also constitutively resistant to clindamycin (cMLS$_B$ phenotype) and carried the *erm*(B) gene (except for one, which harbored *erm*(A) instead of *erm*(B)). The remaining three erythromycin-resistant isolates presented the M phenotype of macrolide resistance (2%), harboring the *mef*(A) gene. There was a significant decrease in the overall prevalence of this phenotype when compared with invasive isolates from 2000-2005 (6%, $P = 0.024$) [18].

Tetracycline resistance was found in 20 isolates (10.5%), representing a decrease, although not significant, in relation to the period of 2000-2005 (17%). All tetracycline-resistant isolates carried the *tet*(M) gene and four also carried *tet*(L). Seven of these isolates presented also the cMLS$_B$ phenotype, but no association between resistance to macrolides and tetracycline could be shown.

Two isolates presented intermediate levofloxacin resistance (MIC = 3 and 4 µg/ml). Both these isolates carried the S79F mutation in the *parC* gene. The *gyrA* gene did not present any alteration in the QRDR when compared with the wild type sequence. One isolate presented high level levofloxacin resistance (MIC > 32 µg/ml). This isolate carried not only the S79F mutation in *parC*, but also the S81F mutation in the *gyrA* gene.

In agreement with the previously studied period, bacitracin resistance among invasive isolates in Portugal was very low ($n = 3$) and was identified solely in isolates presenting the cMLS$_B$ phenotype (Table V-1) [18].

Table V-1 Properties of the 191 GAS isolates collected from invasive infections in Portugal during 2006-2009.

***emm* type (no. of isolates)**	**PFGE cluster[a] (no. of isolates)**	**T type[b] (no. of isolates)**	**SAg genes profile[c] (no. of isolates)**	**ST[d] (no. of isolates)**	**Antimicrobial resistance[e] (no. of isolates)**
1 (56)	A_{83} (53)	1 (53)	3 (6), 10 (45), 44 (2)	28 (18)	S (51), $cMLS_B$ (2)
	P_5 (1)	1 (1)	5 (1)	618 (1)	M (1)
	Other (2)	1 (2)	3 (2)	ND	S (2)
89 (24)	C_{32} (23)	B3264 (22), NT (1)	27 (3), 29 (16), 46 (4)	[101 (5), 408 (2)]	S (22), Lev (1)
	Other (1)	B3264 (1)	46 (1)	101 (1)	S (1)
3 (20)	B_{33} (18)	3(16), NT (2)	8 (18)	[15 (4), 315 (29)]	S (18)
	Other (2)	NT (2)	8 (1), 53 (1)	15 (1)	S (2)
6 (15)	D_{26} (15)	6 (12), NT (3)	2 (14), 51 (1)	[382 (2), 411 (3)]	S (15)
4 (13)	K_8 (7)	4 (7)	23 (7)	39 (2)	S (7)
	J_8 (2)	4 (2)	23 (2)	39 (1)	M (2)
	Other (4)	4 (3), NT (1)	1 (1), 23 (2), 44 (1)	39 (1)	S (3), Tet (1)
12 (10)	E_{21} (10)	12 (9), NT (1)	16 (6), 33 (4)	36 (3)	S (10)
28 (9)	H_{12} (5)	28 (4), NT (1)	24 (3), 27 (1), 54 (1)	458 (1)	S (5)
	N_6 (3)	28 (2), NT (1)	27 (2), 50 (1)	52 (1)	[$cMLS_B$, Bac] (3)
	M_6 (1)	28 (1)	24 (1)	52 (1)	S (1)
87 (6)	O_5 (5)	28 (5)	20 (5)	62 (1)	S (5)
	L_7 (1)	28 (1)	54 (1)	62 (1)	Tet (1)
5 (6)	Other (6)	5/27/44 (2), NT (4)	5 (1), 29 (5)	99 (2)	S (4), Tet (2)
44 (5)	G_{13} (3)	5/27/44 (3)	32 (1), 38 (2)	25 (1)	S (3)
	Other (2)	11 (1), 5/27/44 (1)	12 (1), 56 (1)	25 (1)	S (1), Tet (1)
11 (5)	I_8 (5)	11 (5)	16 (5)	403 (3)	[$cMLS_B$, Tet] (5)
Other[f]	15 PFGE clusters	11 different types[g]	16 different profiles	4 different STs[h]	6 different phenotypes[i]

[a] Clusters are designated by capital letters and a subscript number indicating the total number of isolates in each cluster (considering the whole collection of 351 invasive isolates obtained during 2000-2009). Whenever a PFGE cluster included fewer than 5 isolates of that particular *emm* type, it was not discriminated, and all such isolates were grouped under "other".

[b] NT, non-typeable.

[c] The SAg genes included in each profile are indicated in Table V-2.

[d] A total of 62 isolates from 2006-2009 were analyzed by MLST. ND, not determined. Brackets indicate STs that belong to the same clonal complex defined by the goeBURST algorithm (http://goeburst.phyloviz.net/) with the complete *S. pyogenes* database available at spyogenes.mlst.net.

[e] S, susceptibility to all antimicrobials tested; M, presenting the M phenotype of macrolide resistance; $cMLS_B$, presenting the $cMLS_B$ phenotype of macrolide resistance; Tet, non-susceptibility to tetracycline; Lev, non-susceptibility to levofloxacin; Bac, resistance to bacitracin.

[f] 16 *emm* types identified in < 5 isolates (n = 22), including: *emm*22 (4), 18 (2), 64 (2), 77 (2), 2 (1), 19 (1), 75 (1), 76 (1), 81 (1), 84 (1), 90 (1), 103 (1), 118 (1), st4365 (1), st221 (1), st3857 (1).

[g] 11 T types: NT (9), 12 (3), 13 (2), 1 (1), 2 (1), 3 (1), 22 (1), 23 (1), 25 (1), 5/27/44 (1), B3264 (1).

[h] 4 STs: 28 (1), 46 (1), 99 (1), 619 (1).

[i] 6 antimicrobial resistance phenotypes: S (10), [$cMLS_B$, Tet] (2), $cMLS_B$ (1), Tet (7), [Tet, Lev] (1), Lev (1).

Table V-2 SAg profiles identified in invasive GAS isolates in Portugal during 2000-2005 (P1) and 2006-2009 (P2).

SAg profile	*speA*	*speC*	*speG*	*speH*	*speI*	*speJ*	*speK*	*speL*	*speM*	*ssa*	*smeZ*	No. of isolates (P1/P2)
1	+	+	-	-	-	-	-	-	-	+	+	0/1
2	+	+	+	-	-	-	+	-	-	-	+	11/14
3	+	+	+	-	-	+	-	-	-	-	+	1/8
4	+	+	+	-	-	-	-	+	+	-	+	1/0
5	+	+	+	-	-	-	-	-	-	-	+	1/4
8	+	-	+	-	-	-	+	-	-	+	+	16/19
10	+	-	+	-	-	+	-	-	-	-	+	32/46
11	+	-	+	-	-	-	-	-	-	-	+	5/0
12	-	+	+	+	+	+	-	-	-	+	+	0/1
16	-	+	+	+	+	-	-	-	-	-	+	4/11
17	-	+	+	+	-	-	-	-	-	-	+	2/0
19	-	+	+	-	-	-	+	-	-	+	+	0/2
20	-	+	+	-	-	+	-	-	-	+	+	0/5
21	-	+	+	-	-	-	-	-	-	+	+	1/0
23	-	+	-	-	-	-	-	-	-	+	+	9/11
24	-	+	+	-	-	+	+	-	-	-	+	10/4
26	-	+	+	-	-	-	+	-	-	-	+	2/1
27	-	+	+	-	-	+	-	-	-	-	+	17/7
29	-	+	+	-	-	-	-	-	-	-	+	6/23
30	-	+	-	-	-	-	-	-	-	-	+	0/2
31	-	+	+	-	-	-	-	+	+	-	-	1/1
32	-	-	+	+	+	+	-	-	-	+	+	6/1
33	-	-	+	+	+	-	-	-	-	-	+	7/5
34	-	-	+	+	-	+	-	-	-	-	+	1/0
35	-	-	+	+	-	-	-	-	-	-	+	1/0
38	-	-	+	-	-	+	-	-	-	+	+	0/2
40	-	-	+	-	-	-	-	-	-	+	+	1/0
43	-	-	+	-	-	-	+	-	-	-	+	2/0
44	-	-	+	-	-	+	-	-	-	-	+	1/4
45	-	-	+	-	-	-	-	+	+	-	+	1/1
46	-	-	+	-	-	-	-	-	-	-	+	19/7
47	-	-	-	-	-	-	-	-	-	-	+	1/0
49	-	-	-	-	-	-	-	-	-	-	-	1/0
50	+	+	+	+	+	+	+	-	-	-	+	0/1
51	+	+	+	+	+	-	+	-	-	-	+	0/1
52	+	+	+	-	-	-	+	-	-	+	+	0/2
53	+	-	+	-	-	-	-	-	-	+	+	0/1
54	-	+	+	+	+	+	-	-	-	-	+	0/2
55	-	-	+	+	+	+	-	-	-	-	+	0/1
56	-	-	+	+	-	+	-	-	-	-	-	0/2
57	-	-	+	-	-	+	-	+	+	-	+	0/1

Molecular typing

The 191 invasive GAS isolates analyzed in this study presented a high genetic diversity, with 15 different T types (SID = 0.856 [$CI_{95\%}$ 0.827 to 0.886]), 27 *emm* types (SID = 0.872 [$CI_{95\%}$ 0.840 to 0.903]), 36 PFGE types (profiles with ≥ 80% relatedness in the dendrogram) (SID = 0.886 [$CI_{95\%}$ 0.855 to 0.918]), and 31 SAg profiles (SID = 0.902 [$CI_{95\%}$ 0.878 to 0.927]).

The four most prevalent *emm* types, namely *emm*1, *emm*89, *emm*3, and *emm*6, accounted for 60% of all isolates (Table V-1). The prevalence of *emm*1 and *emm*89, which became the second most frequent *emm* type among invasive GAS isolates in Portugal, increased when compared with the collection of invasive isolates recovered in Portugal between 2000 and 2005 [17], while *emm* types 28 and 64 decreased (Figure V-1). Among the *emm* types identified in ≥ 5 isolates in 2000-2009, all occurred in both time periods, except for *emm*5, which was only detected in 2006-2009. The overall distribution of *emm* types differed significantly between the two time periods (P = 0.029), although none of the differences in individual *emm* types was statistically supported after FDR correction for multiple testing. A new *emm* type was identified in this study, namely *st*4365, which is most similar to *emm* type 5.46 (73.5% identity at the nucleotide level). This *emm* type was identified in only one strain that was clustered into a minor PFGE cluster with three *emm*5 isolates and also presented an ST characteristic of this *emm* type (ST99).

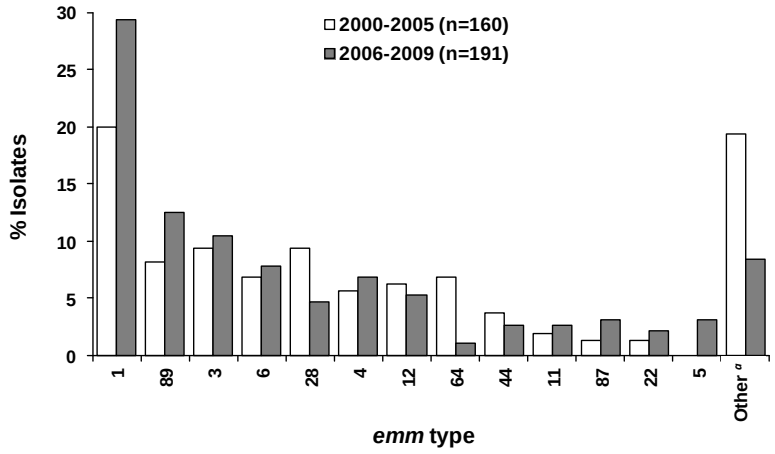

Figure V-1 Distribution of *emm* types among invasive GAS isolates collected from 2000 to 2009. [a]*emm* types identified in < 5 isolates, including *emm*2 (*n*=2), 9 (*n*=2), 18 (*n*=4), 19 (*n*=1), 30 (*n*=1), 43 (*n*=4), 53 (*n*=2), 58 (*n*=3), 74 (*n*=1), 75 (*n*=1), 76 (*n*=1), 77 (*n*=3), 78 (*n*=4), 81 (*n*=1), 84 (*n*=1), 90 (*n*=2), 94 (*n*=1), 103 (*n*=2), 113 (*n*=3), 114 (*n*=1), 118 (*n*=1), st4365 (*n*=1), st106M (*n*=1), st221 (*n*=1), st3857 (*n*=1), stG1750 (*n*=1), stIL103 (*n*=1).

The *emm* typing results were reflected in those of the major PFGE clusters, which also presented significant changes in 2006-2009 when compared to 2000-2005 ($P < 0.001$) (Figure V-2). Considering the whole collection of invasive isolates, obtained between 2000 and 2009, 16 major PFGE clusters were identified, representing 82% of the invasive isolates collected in 2006-2009. PFGE cluster A_{83}, which included mostly macrolide-susceptible isolates characterized by *emm*1-T1-ST28 (Table V-1), remained the dominant invasive lineage in Portugal (Figure V-2), reaching a prevalence of 28% in the most recent period. PFGE cluster C_{32} (*emm*89) also increased, to become the second most prevalent cluster, while the prevalence of PFGE cluster B_{33} (*emm*3) remained constant. A marked decline of PFGE clusters F_{15} (*emm*64) and G_{13} (*emm*44) was also noted. None of these changes maintained significance after FDR correction.

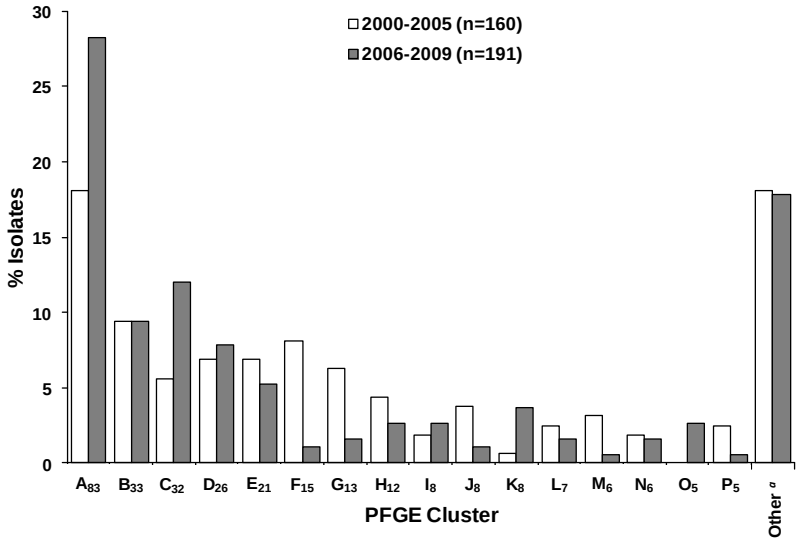

Figure V-2 Distribution of major PFGE clusters among invasive GAS isolates collected from 2000 to 2009. [a] Isolates with unique PFGE patterns or included in clusters of < 5 isolates.

MLST analysis resulted in the identification of 18 different STs (SID = 0.884 [$CI_{95\%}$ 0.823 to 0.944]) among the 62 isolates analyzed, representing 15 distinct *emm* types. Different STs found within the same *emm* type (namely within *emm* types 1, 3, 6, 28, and 89) were always single locus variants (SLVs) (Table V-1). Two new STs were identified in this study – ST618, which included a new *recP* allele (*recP*100), and ST619.

Macrolide and tetracycline resistance was observed in isolates of several genetic lineages (Table V-1). Isolates presenting the $cMLS_B$ phenotype of macrolide resistance were found in five of the major PFGE clusters and belonged to *emm*11 (n = 5), *emm*28 (n = 3), *emm*1 (n = 2), *emm*22 (n = 1), *emm*76 (n = 1), and st3857 (n = 1), while isolates with the M

phenotype were included in PFGE clusters J_8 and P_5, belonging to *emm* types 4 (n = 2) and 1 (n = 1), respectively. Among the 20 tetracycline resistant isolates, which belonged to 14 different *emm* types, only nine were included in four of the major PFGE clusters, while the remaining were found in groups with less than five isolates. The three levofloxacin nonsusceptible isolates belonged to different genetic lineages. From the two isolates presenting intermediate resistance, one was an *emm*81-T23 isolate presenting also tetracycline resistance, and the other was an *emm*77, T-nontypeable isolate. None of them was included in any of the major PFGE clusters. The levofloxacin highly resistant isolate was characterized by *emm*89-TB3264 and belonged to PFGE cluster C_{32} (Table V-1). The three bacitracin resistant isolates identified in this study were all grouped together with the ones isolated in 2000-2005 in PFGE cluster N_6, and were characterized by *emm*28-ST52 (Table V-1).

Diversification of SAg profiles

The chromosomally encoded SAg genes *speG* and *smeZ* were present in 93% and 98% of the isolates, respectively. The *speG* gene was absent solely in isolates belonging to *emm*4 and *emm*77. The *smeZ* gene was absent in the only isolates of *emm*2 and *emm*84 present in the collection, as well as in one of the *emm*44 isolates. In contrast, *speJ*, which chromosomal or phage origin remains controversial, was detected in 45% of the isolates. The prevalence of *speA* (51%) and *speC* (53%) increased among the most recent isolates in relation to the previous period (*speA*, 42%, and *speC*, 41%). The remaining phage-encoded genes, namely *ssa* (24%), *speH* (13%), *speI* (12%), *speK* (23%), *speL* and *speM* (2% each) were detected in similar frequencies to those found in 2000-2005. Globally, 14 SAg profiles were identified only in the most recent time period, while 9 were only present in 2000-2005 (Table V-2). The overall distribution of SAg profiles identified in at least five isolates was significantly different between the two study periods (P < 0.001), although the differences in the prevalence of individual SAg genes or SAg profiles did not retain significance after FDR.

The SID for *emm* typing and PFGE profiling decreased significantly between 2000-2005 and 2006-2009, in contrast to the SID for T typing and SAg profiling that remained constant (Figure V-3). This suggests a diversification of T types and SAg profiles among isolates belonging to the same *emm* type and PFGE cluster. The intra-*emm* diversification of SAg profiles was further supported by a decrease in the predictive capacity of the *emm* type over the SAg profile, as shown by the significant decrease in the respective adjusted Wallace

values ($AW_{emm \rightarrow SAg,2000\text{-}2005}$ = 0.847 [$CI_{95\%}$ 0.774 to 0.919]; $AW_{emm \rightarrow SAg,2006\text{-}2009}$ = 0.622 [$CI_{95\%}$ 0.508 to 0.736]).

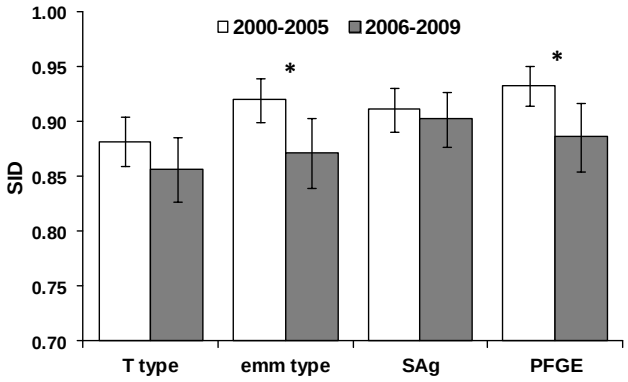

Figure V-3 Simpson's index of diversity of the four typing methods used in the characterization of all strains in the two time periods (2000-2005 and 2006-2009). **P* = 0.01

The SID for the SAg profiles was calculated separately for each *emm* type in each of the two study periods. From 2000-2005 to 2006-2009, the diversity of SAg profiles increased significantly within particular *emm* types, namely *emm*1, *emm*28 and *emm*44 (Figure V-4). The distribution of the distinct SAg profiles identified in each of these *emm* types in the two time periods is presented in Table V-3. The diversification of the profiles occurred mostly through changes in the phage-encoded genes, but in some cases also involved the chromosomal genes *smeZ* and *speJ*. Among *emm*89 isolates, there was no change in the overall diversity of SAg genes, but it was possible to observe a switch in the dominant profile from SAg27 to SAg29 (Table V-2), in which *speJ* seems to have been lost (*n* = 11, SAg27 and *n* = 0, SAg29 isolates in 2000-2005; *n* = 3, SAg27 and *n* = 16, SAg29 isolates in 2006-2009). The additional SAg profiles identified within *emm* types 1, 28, 44, and 89 in 2006-2009 were associated with the same STs previously identified among isolates of the same *emm* type, or with STs that are SLVs of those identified previously. With very few exceptions, these isolates were also grouped into existing PFGE clusters.

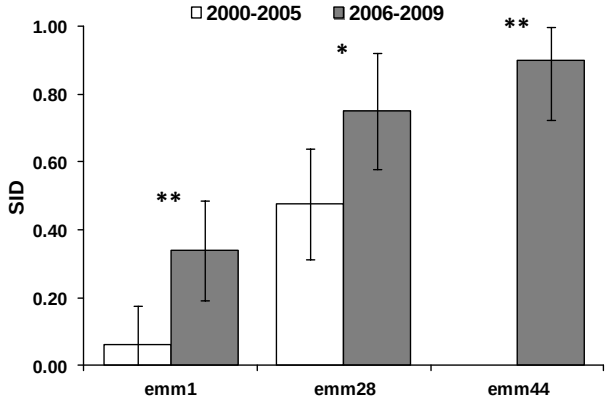

Figure V-4 Simpson's index of diversity of the SAg profile within *emm*1, *emm*28, and *emm*44 isolates, showing significant differences between the two time periods (2000-2005 and 2006-2009). **P* = 0.02; ***P* < 0.01

Table V-3 SAg profiles identified for *emm* types with significant SID differences between the two time periods (2000-2005 and 2006-2009).

	SAg genes (SAg profile)	No. of isolates	
		2000-2005	2006-2009
emm1	*speA, speG, speJ, smeZ* (SAg10)	31	45
	speA, speC, speG, speJ, smeZ (SAg3)	1	8
	speG, speJ, smeZ (SAg44)	-	2
	speA, speC, speG, smeZ (SAg5)	-	1
emm28	*speC, speG, speJ, speK, smeZ* (SAg24)	10	4
	speC, speG, speJ, smeZ (SAg27)	5	3
	speA, speC, speG, speH, speI, speJ, speK, smeZ (SAg50)	-	1
	speC, speG, speH, speI, speJ, smeZ (SAg54)	-	1
emm44	*speG, speH, speI, speJ, ssa, smeZ* (SAg32)	6	1
	speG, speJ, ssa, smeZ (SAg38)	-	2
	speC, speG, speH, speI, speJ, ssa, smeZ (SAg12)	-	1
	speG, speH, speJ (SAg56)	-	1

DISCUSSION

The clonal composition of GAS isolates causing invasive infections appears to present significant fluctuations in some European countries during recent years, although the M1T1 clone remains the dominant lineage in most regions [7, 19, 25, 29, 32, 36, 41]. The increase in virulence and fitness of this and other GAS clones has been associated with the acquisition of SAg genes, their particular variants and other virulence factors through horizontal gene transfer events, which constitute the main mechanism driving genetic diversity in *S. pyogenes* [27, 47, 50]. In order to identify possible shifts in the clonal structure of the isolates responsible for invasive GAS infections in Portugal and in the genomic diversity of the major clones, we characterized a collection of GAS strains isolated from normally sterile sites during 2006-2009 and compared their phenotypic and molecular properties with those of invasive isolates recovered during 2000-2005 [17, 18].

The erythromycin resistance rate among invasive GAS isolates collected in Portugal between 2006 and 2009 (8%) was not significantly lower than the one reported for 2000-2005 [18]. The value is in line with recent reports from countries like France, Greece, Poland, and Canada [22, 36, 45, 48], although resistance rates above 20% have been reported in other Southern European countries, like Spain and Italy [6, 39]. The majority of macrolide-resistant isolates analyzed in this study was also constitutively resistant to clindamycin, resulting in an overall clindamycin resistance rate (7%) similar to the one of the previous study period (6%) [18]. This phenotype ($cMLS_B$) was mostly associated with the presence of the *erm*B gene, which was detected for the first time among *emm*1 isolates in Portugal. These isolates were included in the PFGE cluster dominated by macrolide-susceptible *emm*1 isolates, suggesting a recent acquisition of this gene. Previously, macrolide resistance among *emm*1 isolates from Portugal had only been found in a distinct genetic lineage expressing the M phenotype associated with the presence of *mef*(A), found among invasive infections, pharyngitis, and carriage [18, 35, 43].

In the current study, reduced susceptibility to levofloxacin is reported for the first time among *S. pyogenes* isolates recovered from invasive infections in Portugal, although with a low prevalence (2%). These isolates presented mutations that were previously associated with decreased fluoroquinolone resistance in GAS and other streptococci [30, 33] and consistent with their MICs. Remarkably, neither the two non-susceptible isolates, nor the highly resistant isolate presented the *emm* types most commonly associated with reduced fluoroquinolone

resistance in GAS. This suggests that the clonal spread of fluoroquinolone non-susceptible GAS isolates observed in other countries [26, 30, 37] is not occurring among invasive GAS isolates in Portugal and that fluoroquinolone resistance in *S. pyogenes* remains rare, in contrast to its high prevalence among the contemporary and closely related *Streptococcus dysgalactiae* subsp. *equisimilis* isolates causing human infections [33].

Our previous studies identified a clone of macrolide-susceptible *emm*1-T1-ST28 isolates carrying the SAg genes *speA, speG, speJ,* and *smeZ* as the most prevalent among a genetically diverse collection of invasive GAS isolates collected during 2000-2005 [17, 18]. This clone was overrepresented among invasive GAS isolates when compared with a collection of pharyngeal isolates from the same geographic region and time period, indicating its high invasive disease potential [18]. The *emm*1-T1-ST28 clone remained clearly dominant, even increasing in frequency from 18% in 2000-2005 to 28% in 2006-2009. This trend is in agreement with recent reports from Finland and Spain [29, 41], whereas in Germany and France a decrease in the frequency of *emm*1 isolates has been observed [19, 36]. The clone *emm*89-TB3264-ST101 also increased in frequency from 6% to 12% between the two time periods and became the second most prevalent clone in the present study. *emm*89 was the fourth most prevalent *emm* type in a large European study, with an overall frequency of 8% [25], but presented a higher prevalence in Sweden (16%) where it was the dominating *emm* type in a study from 2002-2004 [9]. In Spain, this *emm* type emerged in 2002-2005 and showed an increasing trend since [29].

In a previous study, we identified a clone representing *emm*64-ST164 isolates that does not carry any of the phage-encoded SAg genes as being significantly associated with invasive infections in Portugal [18]. However, this clone seems to be decreasing in Portugal and was identified only in two isolates in the present study. This suggests that, in contrast to the highly successful M1T1 clone that remains the most prevalent among invasive infections in most developed countries, the clone *emm*64-ST164 emerged briefly in Portugal, but did not persist.

Our data indicate that some of the clones causing invasive GAS infections in Portugal are going through a process of diversification of their SAg profiles. This conclusion is supported by (i) a lower capacity of the *emm* type to predict the SAg profile in this study when compared to the period of 2000-2005; (ii) a lower diversity of *emm* and PFGE types in the most recent collection associated with a constant diversity of SAg profiles; and (iii) a statistically significant increase in the diversity of the SAg profiles associated with the dominant *emm* type (*emm*1), as well as with *emm* types 28 and 44. Among isolates presenting

*emm*89, the second most frequent *emm* type among recent invasive isolates in Portugal, this increase in SAg profile diversity was not observed, but there was a shift in the dominant SAg profile associated with this *emm* type, compatible with the loss of the *speJ* gene. The data available so far do not allow us to confidently conclude if this change in SAg repertoire is associated with an increase in the prevalence of this *emm* type in invasive GAS infections.

The isolates with the new SAg profiles were mostly grouped into the same PFGE clusters of the isolates sharing the same *emm* type recovered in 2000-2005 and belonged to the same or to closely related STs, suggesting that the new SAg profiles have emerged recently from isolates belonging to the same genetic lineages. The intra-*emm* type diversification of SAg profiles can be interpreted as being associated with both acquisition and loss of SAg genes, mostly of phage-encoded genes, but also of *speJ*, which is generally considered to be part of the bacterial core genome. However, this gene resides in a locus presenting evidence of mobile genetic elements related events, which may explain its variable presence among GAS lineages [27]. Together with the recent acquisition of resistance determinants, these data document ongoing evolution of the GAS population causing invasive disease where horizontal genetic exchange plays an important role.

The occurrence of new SAg combinations among previously identified GAS clones observed in this study may be the result of genetic drift, reflecting the recently published data documenting the short-term stability of the SAg gene complement of *S. pyogenes* isolates recovered in Portugal [16]. However, it may also indicate an increase of genomic plasticity of the invasive isolates, despite an overall decrease in the diversity of the circulating genetic lineages, in response to changes in host imposed selective pressures. This genomic plasticity may result in the emergence of new variants with enhanced fitness, invasive capacity, or specific tissue adaptation. Continued surveillance of the clonal structure and SAg gene content of invasive GAS isolates may therefore afford new insights into the dynamics of the evolution of the *S. pyogenes* population responsible for severe infections in temperate climate regions.

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CHAPTER VI

GENERAL DISCUSSION

Invasive infections caused by *S. pyogenes* are recognized as an important cause of morbidity and mortality worldwide [4]. It is bewildering how the same organism that may asymptomatically colonize the human host or cause mild superficial infections, like pharyngitis and impetigo, can also invade deeper tissues and lead to an extremely rapid progression of very severe infections. Notwithstanding the role that individual characteristics of the human host, in particular of the immune system, can play in disease outcome [26], there has long been a general notion that certain bacterial characteristics and the presence of particular virulence factors must be determinant for the ability of GAS to cause invasive disease. This would result in the association of specific GAS clones with severe infections, when compared with mild disease presentations and asymptomatic carriage.

The resurgence of invasive GAS infections since the late 1980s in developed countries led to a growing interest in the study of the isolates causing these infections worldwide. The work presented in this thesis provides the first detailed study focusing specifically on invasive GAS isolates in Portugal. The genetic lineages associated with invasive GAS infections throughout ten years were extensively characterized and compared with the situation observed in other countries, as well as with the clonal structure of the GAS population causing pharyngitis in Portugal. The characterization of the isolates involved a variety of phenotypic and molecular typing methods, including a newly developed PCR-based method to efficiently determine the SAg gene content of each strain.

Antimicrobial resistance of invasive GAS isolates in Portugal

The antimicrobial treatment of choice for severe GAS infections consists on the association of penicillin and clindamycin, while vancomycin, linezolid, daptomycin, or quinupristin/dalfopristin are recommended alternatives for penicillin-allergic patients [46]. In the present work, all studied GAS isolates were tested for susceptibility to a number of antimicrobial agents, of clinical or epidemiological relevance. All the tested isolates were susceptible to penicillin, chloramphenicol, vancomycin, linezolid, and quinupristin-dalfopristin.

The erythromycin resistance rate among the invasive isolates was 12% in the period of 2000-2005, a value significantly lower than the one determined for pharyngitis isolates in the same time period ($P = 0.016$) [23]. In 2006-2009 macrolide resistance decreased, although not significantly, to 8% [21], possibly reflecting the decreasing trend observed among isolates associated with pharyngitis in Portugal [44]. While in 2000-2005 the two main macrolide-

resistance phenotypes were nearly equally prevalent, in 2006-2009 there was a significant decrease in the number of isolates presenting the M phenotype (P = 0.024), leading to a dominance of the $cMLS_B$ phenotype (81%). In 2000-2005, both macrolide resistance phenotypes were limited to a small number of PFGE clusters and *emm* types (*emm*4 and *emm*1 for the M phenotype; *emm*28 for $cMLS_B$ phenotype), mostly mirroring the clonal structure of the pharyngeal macrolide-resistant isolates. This observation was maintained for the M strains recovered during 2006-2009, while the $cMLS_B$ isolates from this period were dispersed by six different *emm* types, the most common of which was no longer *emm*28, but *emm*11. These results are suggestive of a possible acquisition of macrolide-resistance genetic determinants (in particular the *erm*(B) gene) by a larger number of lineages causing invasive infection in Portugal, including the dominant *emm*1-T1-ST28 PFGE clone, which in the previous time period included only macrolide-susceptible isolates [23]. Nevertheless, the genetic determinants associated with macrolide resistance remained the same and were also the ones identified among the isolates causing pharyngitis (*mef*(A) in the case of isolates presenting the M phenotype and *erm*(B) in $cMLS_B$ isolates). Several studies demonstrate that the M1T1 lineage has evolved through a number of recombination events, including the sequential acquisition of MGEs carrying genetic traits that may have contributed to its evolutionary success [29, 47]. The high prevalence of this clone in the population may enhance the probability of M1T1 strains participating in horizontal gene transfer events and acquiring new virulence and resistance determinants. The acquisition of the *mef*(A) and *erm*(B) genes, both located on MGEs [50], may further contribute to the persistence of this lineage in the population by conferring resistance to macrolides, which are commonly used in the treatment of respiratory infections. However, the limited expansion of macrolide-resistant M1T1 isolates may indicate that their lineage is not adapted to carrying the genetic elements harboring these genes that may interfere with the properties that make this clone so successful.

In contrast to the situation observed with macrolides, tetracycline resistance was higher among the invasive isolates from 2000-2005 than in the pharyngitis subset ($P < 0.001$), mostly due to a higher prevalence of tetracycline-resistant *emm*64 isolates among the invasive group [23]. The decrease in the prevalence of this clone contributed to a lower proportion of tetracycline-resistant isolates in the period of 2006-2009 (10.5%), when compared with the previous time period (17%) [21]. Tetracycline resistance was spread by a much larger number of PFGE clones and *emm* types than macrolide resistance and was mostly associated with the presence of the *tet*(M) gene, both in invasive and pharyngitis isolates.

The clonal spread of GAS strains presenting reduced fluoroquinolone resistance reported in other countries was not observed among invasive isolates in Portugal. Three levofloxacin non-susceptible invasive isolates (one highly resistant and two with reduced susceptibility) were recovered in the period of 2006-2009, presenting three different *emm* types, none of which matched the *emm* types commonly associated with fluoroquinolone non-susceptibility [30, 51]. Levofloxacin non-susceptibility has been previously reported, albeit at a low frequency, among *S. pyogenes* isolates recovered from carriage and non-invasive infections in Portugal [39], while a large longitudinal study of GAS isolates associated with tonsillo-pharyngitis in Portugal did not identify any levofloxacin non-susceptible isolates between 1999 and 2007 [33]. Remarkably, levofloxacin non-susceptibility is much lower among *S. pyogenes*, when compared with *S. dysgalactiae* subsp. *equisimilis* isolates recovered in Portugal, despite the evidences for a global pool of *parC* and *gyrA* genes shared between the two species [38].

Bacitracin resistance among invasive isolates was found in only one PFGE cluster, characterized by *emm*28-T28-ST52 [21, 23], mostly in agreement with the results obtained for isolates associated with oropharyngeal carriage, tonsillo-pharyngitis, and skin and soft tissue infections in Portugal [40, 45]. Together, these studies show that bacitracin resistance among the circulating GAS isolates in Portugal is low (< 5%) and essentially limited to one genetic lineage, but must be taken into account when using the bacitracin-susceptibility test for the identification of *S. pyogenes* strains from clinical specimens.

Molecular epidemiology of invasive GAS isolates in Portugal

The GAS isolates causing invasive infection in Portugal throughout the 2000s decade were characterized by a high genetic diversity, as attested by the high SID values obtained for most of the typing methods in both time periods studied [21–23], including lineages that are recognized to be widely disseminated worldwide, and lineages that apparently present a more limited geographic spread.

In agreement with the situation in other developed countries [9, 25, 34, 36], there were fluctuations in the clonal composition of the invasive isolates in Portugal during the last decade, notwithstanding the dominance of a clone composed of macrolide-susceptible isolates characterized as *emm*1-T1-ST28 and carrying the genes *speA, speG*, *speJ*, and *smeZ*. Notably, this clone was found to be significantly associated with invasive infections in Portugal, like in some other countries [8, 13, 14, 28, 51], supporting the generalized notion that the M1T1

clone has an enhanced invasive potential [23]. However, the present study further characterized this invasive lineage by showing that *emm*1-T1-ST28 isolates are clustered into two distinct PFGE clusters according to macrolide resistance, and only the macrolide-susceptible clone presented a significant association with invasive disease. One other PFGE cluster, composed mainly of tetracycline-resistant isolates characterized by *emm*64-ST164 and carrying the SAg genes *speG* and *smeZ*, was also found to be overrepresented among invasive isolates in Portugal during the period of 2000-2005 [23]. This clone, in contrast to the *emm*1-T1-ST28 lineage that has remained for several years the dominant clone causing invasive infections in different world regions, is not frequently reported among invasive isolates in other countries, and it nearly disappeared in Portugal in the period of 2006-2009 [21]. These results emphasize the possible emergence of highly invasive clones with a limited geographic and temporal spread. Although these two clones accounted for more than 25% of the isolates causing invasive infections in Portugal during 2000-2005, the majority of the invasive GAS isolates in Portugal seem to reflect the clonal structure of the general circulating GAS population [23].

The comparison of the clonal composition of the invasive isolates recovered during the two studied time periods suggests an overall decrease in the diversity of the circulating invasive GAS lineages [21]. However, the data also indicate an ongoing genetic evolution of some of these lineages. This was evidenced by the acquisition of antimicrobial resistance determinants and by the diversification of the SAg gene content in isolates sharing the same *emm* type, T type, PFGE cluster, and the same or closely related STs. These observations, further support horizontal gene transfer mechanisms as the main contributors to genomic diversification in GAS [32], since both the transfer of macrolide resistance determinants and the loss and acquisition of SAg genes are mostly mediated by the lateral transfer of MGEs [31, 50]. Nevertheless, our studies also illustrate the possible occurrence of spontaneous mutations affecting genes with a recognized role in virulence, evidenced by the deletions in the chromosomal region encoding SpeB, SpeF, and Rgg that were detected in both invasive and non-invasive isolates, although at a very low frequency [20].

Despite the several efforts to develop a GAS vaccine capable of producing an effective immunological response against all strains, the M protein-based 26-valent vaccine is the only one that has reached phase II clinical trials [10]. According to the *emm* types identified among the isolates recovered from normally sterile sites in the present study, this vaccine was estimated to cover 69% of the isolates causing invasive infections during 2000-2005 [22]. The potential coverage of this vaccine increased to 82% in the period of 2006-2009, mostly due to

the decrease of *emm*64 and of a number of other non-vaccine *emm* types that were represented by a few isolates each. However, following the publication of studies from several countries reporting lower potential vaccine coverage than initially expected, a new 30-valent M protein vaccine has been recently proposed [11]. In the overall collection of invasive isolates recovered during 10 years in Portugal, the 30-valent vaccine would potentially increase the coverage from 76% to 89%, mostly due to the inclusion of *emm*4 and *emm*44 in the new formulation. However, these estimates are of limited value, since they are based solely on the M types included in the vaccines and the effective coverage rates may be considerably different. The 30-valent vaccine has been shown to evoke cross-opsonic bactericidal antibodies against several non-vaccine serotypes in rabbits, which may result in a significant increase of the actual vaccine coverage [11]. On the other hand, the vaccine elicited low levels of antibodies against *emm*44 and *emm*77, questioning its protection against these types, which were both identified among invasive isolates in our study.

Molecular markers of increased or decreased invasive capacity of GAS

The nasopharyngeal mucosa is usually considered as the main source of GAS isolates causing invasive infections [7, 16]. However, it remains a matter of debate if the strains causing serious invasive GAS disease reflect the clonal composition of the circulating GAS population, or if they represent clones with enhanced virulence, since different studies comparing invasive and non-invasive isolates have reached conflicting conclusions [8, 12, 13, 34, 41, 42, 51]. In order to identify possible markers for invasiveness among GAS isolates circulating in Portugal, we compared the molecular properties of the genetically diverse collection of invasive GAS isolates recovered during 2000-2005 [22] with a large collection of randomly selected isolates recovered from pharyngitis during the same time period [23].

As mentioned above, two PFGE clusters were significantly overrepresented among invasive infections, indicating a higher invasive potential. In contrast, a PFGE cluster defined by *emm*4-T4-ST39, harboring *speC*, *ssa*, and *smeZ*, and presenting macrolide susceptibility, was significantly associated with pharyngitis, indicating a possible decreased capacity to cause invasive infections.

The associations between PFGE clusters and infection type reflected the occurrence, in these clusters, of molecular properties that were also individually associated with disease presentation. Hence, *emm*1, *emm*64, *speA*, and *speJ* were identified as independent markers for invasiveness, while *emm*4, *emm*75, *ssa*, *speL*, and *speM* were each individually associated

with pharyngitis. As expected, pairwise combinations of some of these characteristics were also found to be significantly associated with either invasive infections or pharyngitis, but no synergistic or antagonistic interactions could be detected between them.

Comparison of typing methods

The technological advances in the field of high throughput sequencing observed in recent years have led to a growing use of whole genome-based methods for epidemiological purposes and evolutionary studies, with several examples of the application of WGS to the study of GAS isolates being found in the literature [2, 3, 17, 18, 29]. WGS approaches are therefore currently considered to have the potential to become the ultimate methodology for bacterial identification and typing. However, there are still important challenges to be addressed regarding the creation of standardized pipelines to efficiently handle and analyze the large amounts of data generated, as well as to integrate the genome sequence data with epidemiological and typing information from different sources [6]. Hence, more conventional typing techniques are expected to remain, at least in the near future, the methods of choice for the routine typing of large GAS collections with surveillance and epidemiological purposes in most laboratories. It is therefore important to take into account the advantages and limitations of the several typing methodologies in use, according to the specific aims of a given study.

Many different typing methodologies have been proposed for *S. pyogenes*, but *emm* typing is currently the gold standard of GAS molecular typing and many studies rely essentially on this method for epidemiological purposes. It has an excellent typeability, with the advantage of allowing an easy comparison of results between different laboratories. However, using a collection of macrolide-resistant GAS isolates associated with pharyngitis, it was previously shown that *emm* typing presents limitations for the accurate identification and characterization of GAS clones and should be complemented with either PFGE or MLST [5]. The present work extended these conclusions to a highly diverse collection of GAS that included both macrolide-resistant and -susceptible isolates recovered from pharyngeal exudates and several normally sterile sites. The limitations of *emm* typing are reflected in the higher ability of PFGE, MLST, and SAg profiling to predict the *emm* type when compared with the reciprocal relationships, as attested by the respective AW coefficients obtained for the 480 isolates analyzed in Chapter IV, as well as for the entire collection of 671 GAS isolates analyzed in this thesis (Figure VI). Moreover, *emm* typing presented a lower discriminatory power than PFGE, SAg profiling, and MLST, translated by a lower SID value.

In spite of the technical problems associated with T typing (Chapter I, section 5.1), the interest in this methodology was renewed since the identification of the T antigen as the main structural component of pilus-like structures, which may be involved in tissue tropism, virulence, and protective immunity [35]. However, in the present work, T typing presented a lower discriminatory power, as well as a lower ability to predict the remaining molecular properties, when compared with the other typing methods analyzed (Figure VI) [20]. Moreover, the association of T typing with *emm* typing did not improve the concordance with PFGE and SAg profiling, when compared with *emm* typing alone. The development of a molecular-based method for typing the T antigen chromosomal region may overcome the methodological issues of T serotyping and improve the typeability of the method, possibly making it more informative in the context of GAS epidemiological typing. However, further improvements to the proposed PCR-based typing scheme [15] are necessary, since it does not seem to cover all the known T serotypes and the non-typeable isolates, nor to have been fully validated against a large collection of the currently phenotipically recognized T-types.

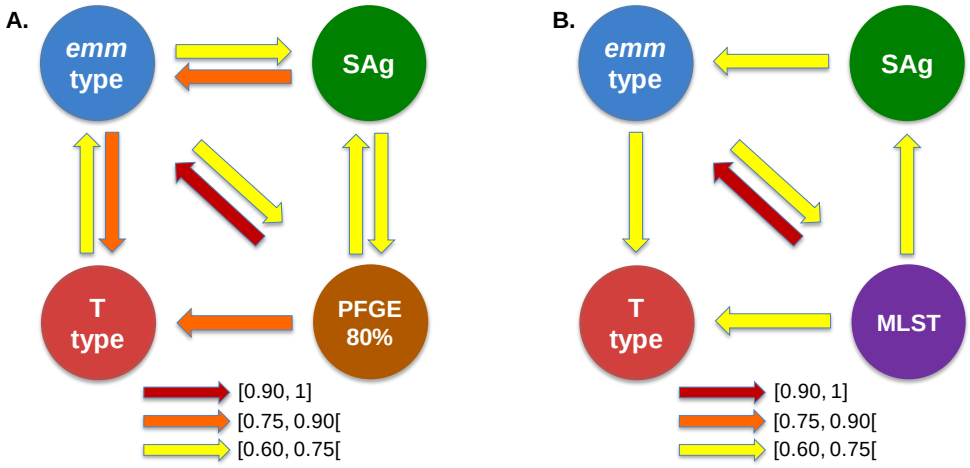

Figure VI Representation of correspondences between the typing methods used to characterize (A) the entire collection of 671 GAS isolates analyzed in the studies presented in Chapters II-V, and (B) the subset of 232 isolates that were analyzed by MLST. PFGE is not included in panel B, since in this subset the correspondence between PFGE and the remaining typing methods is biased due to the criteria used for the selection of the isolates, as explained in Chapter IV. The arrows represent Adjusted Wallace coefficients ≥ 0.60.

The diversity of primers used by different authors for PCR-screening of the SAg genes, associated with a confusing gene nomenclature, has complicated the analysis and comparison of results obtained in different studies. In Chapter II, a simplified SAg profiling scheme addressing only four phage-encoded genes was used, but 16% of the analyzed isolates did not present any of the SAg genes tested [22]. This result, together with a growing number of studies reporting the absence of chromosomal genes in some GAS lineages, prompted us to

extend the PCR screening to the 11 known SAg genes. Therefore, a method for efficiently amplifying all the known allelic variants of the 11 SAg genes using two multiplex PCR reactions was developed [20]. This led to an increase in the discriminatory power of the method, and the chromosomal SAg genes were shown to contribute significantly to the diversity of the SAg repertoire of GAS isolates. The SAg screening approach used in this and most other studies analyzing large GAS collections has the limitation of only identifying the largest number of isolates that have the genetic potential to be able to express the tested SAgs, and does not evaluate the effective production of the exotoxins by the isolates. This limitation must be taken into account when trying to establish correlations between the presence or absence of SAg genes and the virulence of the isolates. Some strains may harbor mutations affecting protein function or expression, as is the case of the characteristic *smeZ* allele of *emm*3 isolates [49]. On the other hand, the regulation of the expression of virulence factors in GAS depends on complex interactions between several regulatory factors and mechanisms, and may vary in different infection stages and distinct GAS lineages. An example is the case of SpeA and SpeB, which have been shown to be involved in a phenotypic shift from $SpeA^{-}$ $SpeB^{+}$ to $SpeA^{+}SpeB^{-}$ during the onset of infection by M1T1 isolates in a murine subcutaneous chamber model [1]. Nevertheless, the present work demonstrates that SAg gene profiling by multiplex PCR, as developed in this study, is a simple, cost-effective complementary typing method that may help to further discriminate and identify GAS clones, since it translates a shorter time-scale of genetic evolution than the other molecular properties commonly used for GAS typing. The importance of SAg profiling is further supported by the association of specific SAg genes with either increased or decreased invasiveness [23], and by the fact that SAg genes are markers for the presence and transfer of prophages that may encode other genes with relevant roles in GAS virulence or tissue tropism. The analysis of the SAg gene content of large collections of GAS isolates may thus contribute to a better understanding of the dynamics of virulence factor transmission among GAS strains and to elucidate the emergence and diversification of virulent clones.

Despite being a labor-intensive method with serious limitations regarding result portability, PFGE was, in our studies, the only method capable of differentiating GAS clones of isolates sharing the same *emm* type, T type, ST, and SAg profile according to macrolide-resistance and disease presentation [23], highlighting the relevance of PFGE when trying to assess the invasive capacity of GAS isolates based on molecular typing. This fact may be explained by the frequent exchange of MGEs between GAS isolates and by the ability of PFGE to differentiate strains that vary in the content of these elements, which frequently carry

resistance determinants and virulence factors. In the context of epidemiological surveillance or investigation, this ability may represent an advantage over MLST, which discriminates the isolates based on the variation of slowly-evolving genes and may thus fail to distinguish isolates from species where horizontal gene transfer plays an important role in genetic diversification, like *S. pyogenes*.

Concluding remarks and future perspectives

The work presented in this thesis, together with reports from other countries, offers new insights into the global epidemiology of the invasive infections caused by GAS, as well as into the population dynamics of this pathogen. While highlighting the importance of using multiple typing methods for the unambiguous discrimination of GAS lineages, the results support the relevance of horizontal gene transfer mechanisms in the genetic diversification of *S. pyogenes* and underscore the influence of the bacterial genetic characteristics in disease outcome. The data also indicate that the invasive GAS isolates do not constitute a stable population, suggesting an ongoing genetic evolution of some major lineages and revealing shifts in the clonal structure of invasive *S. pyogenes* that can have an impact on the treatment and prevention strategies that are currently in use or under development. Therefore, a continued molecular and epidemiological surveillance of invasive GAS isolates in different world regions will allow the monitoring of the emergence of clones with important properties, like antimicrobial resistance and enhanced virulence or fitness, ultimately contributing to assess the adequacy and support the improvement of the approaches used in the clinical management of invasive GAS disease.

Many important questions regarding the pathogenesis and population dynamics of invasive *S. pyogenes* remain unanswered and others emerged from the results obtained in the present work in association with recently published data. Although specific *emm* types and SAg genes are significantly associated with invasive infections, they seem to be only epidemiological markers of invasiveness, but not to fully explain the enhanced virulence of certain clones. In other words, their presence seems not to be necessary and sufficient for the development of invasive disease. On the other hand, a very large fraction of the invasive GAS population mirrored the clonal structure of the GAS isolates causing pharyngitis in Portugal, suggesting that other, unidentified factors are playing a role in the infection process. The increasing availability of cost-effective NGS technologies will probably make WGS methods the strongest contributors to the identification of bacterial genetic factors involved in

invasiveness, by allowing the comparison of large collections of invasive and non-invasive GAS isolates at the complete genome level. It could be fruitful to use this type of approach to compare the macrolide-resistant and -susceptible isolates belonging to *emm*1 and *emm*4 in our collection, in order to better understand if the fact that only the susceptible clones are associated with disease presentation can be explained by genetic differences between the lineages, or if the similar distribution of the resistant isolates into the two infection types simply reflects the antibiotic selection pressure, regardless of the invasive ability of the clone.

The lack of the *hasABC* operon, encoding the genes necessary for the hyaluronic acid capsule biosynthesis, has recently been reported in *emm*4 isolates [19]. Since the hyaluronate capsule is one of the major GAS virulence factors and has been directly implicated in the development of invasive infections [52], it is tempting to speculate that the lack of capsule production may be in the origin of the lower propensity of *emm*4 isolates to cause invasive infections observed in this and other studies [23, 27]. In this regard, it would be interesting to confirm the absence of the capsule operon in both our *emm*4 clones (macrolide-susceptible and -resistant) and perform virulence studies to further clarify the impact of this absence in the pathogenesis of this lineage.

The interaction of *S. pyogenes* with the human host is a multi-factorial process, whose outcome may depend on host factors, on the genetic complement of the bacterium and its repertoire of virulence factors, and on a complex regulatory network controlling the expression of these virulence factors throughout the infection stages. The spontaneous acquisition of mutations in the genes encoding the TCS CovR/S has been implicated in the dissemination of the bacteria into deeper, normally sterile tissues due to the consequent upregulation of virulence factors like the hyaluronic acid capsule, NADase, SpyCEP, Sda1, streptolysins, streptokinase, and some SAgs, and to the downregulation of the protease SpeB, which degrades several virulence factors produced by GAS [48]. Mutations in this regulatory system, as well as in the stand-alone regulator RopB, which positively regulates SpeB, have been shown to result in reduced virulence levels in murine models of GAS infection and the occurrence of mutations in these regulators has been shown to be significantly associated with the development of STSS [24, 48]. However, the definite role of these mechanisms for the outcome of human infections caused by diverse GAS genetic lineages remains unclear, with some conflicting results being found in the literature. For instance, in contrast to the abovementioned results [24], a higher rate of *ropB* polymorphisms resulting in reduced SpeB production has been recently reported among M3 isolates associated with pharyngitis when compared with invasive infections [37], although a higher rate of *covR*/*S* alterations, which

are also expected to downregulate *speB*, was observed in the invasive M3 isolates [43]. The comparison of the genetically diverse collection of invasive and pharyngitis GAS isolates characterized in the present work regarding the genetic sequence of these regulators and the production levels of the key virulence factors that they control may, in the future, afford new insights into the role of these regulatory pathways for the virulence of GAS isolates of diverse genetic backgrounds.

The evasion to the host immune system is considered one of the determinant aspects of the pathogenesis of *S. pyogenes*, and many virulence factors have been implicated in this process (Chapter I, section 4). Therefore, an increased or decreased ability to neutralize specific host defense mechanisms can be one of the key characteristics differentiating invasive and non-invasive GAS isolates. In particular, M1T1 strains have been shown to present an enhanced capacity to evade NETs due to the presence of the phage-encoded DNase Sda1. However, it is still unclear if other DNases produced by lineages that do not harbor this prophage can have a similar role in NET evasion. Isolates associated with invasive and non-invasive infections can be compared with regard to their ability to resist total neutrophil killing and NET-mediated entrapment and killing in particular, and the resulting data can be correlated with bacterial genotypic and phenotypic characteristics. The study of the collection of isolates characterized in this thesis using this kind of approach may shed a new light on the current knowledge concerning the bacterial factors involved in phagocytosis resistance and NET escape, and the importance of these mechanisms for the invasiveness of GAS isolates from diverse genetic contexts.

The results presented in this thesis suggest new questions that point to challenging future directions of work. However, as new GAS virulence factors and regulatory mechanisms are unraveled, and improved molecular biology and bioinformatics tools are developed, many other research avenues will most certainly emerge and lead to a continued progress in the knowledge of this important pathogen and its interaction with the human host.

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