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CHARACTERIZATION OF *STREPTOCOCCUS PYOGENES* ASSOCIATED WITH TONSILLO-PHARYNGITIS IN PORTUGAL WITH PARTICULAR EMPHASIS ON MACROLIDE RESISTANCE

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To my family

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SUMMARY

Keywords: *Streptococcus pyogenes*, pharyngitis, macrolide resistance, intrahost variation, scarlet fever

There are large geographical and temporal variations in the frequency of macrolide-resistant *Streptococcus pyogenes*, as well in the associated resistance phenotypes. Although decreases in macrolide resistance were often associated with low macrolide consumption, natural fluctuations of macrolide resistant clones were also suggested to play an important role in the prevalence of resistant isolates and of resistance phenotypes.

The recent report of heterogeneity found among group A streptococcus (GAS) isolates recovered from the same patient raised the question of whether the strategy used in the microbiology laboratory, the study of microorganisms from a single colony, was leading to the underestimation of macrolide resistance rate. For that purpose, 321 GAS isolates, recovered from 35 pharyngitis patients from one hospital were screened for potential differences in antimicrobial resistance profiles and *emm* types of up to 10 isolates recovered from the same sample. In our collection, all the isolates recovered from the same patient presented an identical antimicrobial resistance profile and *emm* type, indicating that the single colony approach was suitable for a correct identification of macrolide resistance rates and other epidemiological studies.

In this thesis, the epidemiology of macrolide resistant GAS in Portugal is divided in 3 periods - 1998 to 2003, 2004 to 2006 and 2007 to 2011. In all periods, macrolide resistance rates and macrolide resistance phenotypes and genotypes were determined; molecular characterization of the resistant population was accomplished by T typing, *emm* typing, pulsed field gel electrophoresis (PFGE) profiling and multilocus sequence typing (MLST). In the first period, macrolide resistance was high (27%) and stable. However, this stability was accompanied by a variation in the prevalence of the resistance phenotypes. In just 6 years, there was a complete inversion in the prevalence of macrolide resistance phenotypes. The molecular characterization of 325 resistant isolates revealed a diverse population, and the genetic lineages identified in Portugal were also identified in other European countries; the most common were T12-*emm*22-ST46, T28-*emm*28-ST52, T4-

*emm*4-ST39, T12-*emm*12-ST36 and T1-*emm*1-ST28. This unusual situation motivated continuing the surveillance studies. Results from the analysis of the isolates recovered in 2004-2006 allowed the identification of a decreasing trend in macrolide resistance that had started in 1999. The diversity in the clonal composition was still evident among the 156 isolates studied and, with minor exceptions, the same genetic lineages were identified, although differences in their prevalence were noted. Among the most prevalent lineages were T28-*emm*28-ST52, T4-*emm*4-ST39, T11-*emm*11-ST403, and T12-*emm*22-ST46. In order to determine if this decline in macrolide resistance was sustained, the susceptibility of isolates recovered in 2007-2011 was determined and the clonal composition of the resistant population (n=139) was analyzed. The results showed that macrolide resistance continued to decline, reaching in 2011 the lowest value ever recorded in Portugal (2%). Again, macrolide resistant genetic lineages had been previously described in Portugal and other countries, with some changes in their prevalence. The lineage defined by T11-*emm*11-ST403 was now the most prevalent, followed by T12-*emm*22-ST46, T12-*emm*12-ST36 and T28-*emm*28-ST52.

One of the most intriguing observations was that this situation was paralleled by high macrolide consumption, emphasizing the critical role of the fluctuations in the resistant clones for the prevalence of resistance.

Although a small group of resistant lineages has been shown to be widely disseminated, little is known about the reasons for their success and their origin - ongoing acquisition of macrolide resistance genes followed by local spread or simple geographic dissemination. In order to see if the macrolide resistant GAS population was mirroring the general pharyngeal population or if their dynamics were independent, a collection of 803 isolates, representing 50% of all isolates recovered from tonsillo-pharyngitis patients in Portugal from 2000 to 2005 was analyzed by all the typing methods mentioned above. This comparison allowed the identification of specific *emm* types and PFGE clones associated with each of the macrolide resistant phenotypes. Among the *emm* types found in this collection, *emm*4, *emm*22 and *emm*28 were associated with macrolide resistance and *emm*3, *emm*6, *emm*87 and *emm*89 with macrolide susceptibility. Furthermore, the usefulness of PFGE in typing *S. pyogenes* was reinforced with this work since it was the only method capable of differentiating macrolide resistant and susceptible isolates of the same *emm* types. Examples of this could be found in *emm*4 and *emm*1. Additionally, both

populations presented different diversities indicating that macrolide resistant GAS has its own dynamics, independent of the changing patterns of the general population.

The production of the exotoxins SpeA and SpeC was for a long time believed to be responsible for scarlet fever, one of the infections caused by *S. pyogenes.* However, the literature is contradictory and some studies failed to detect such associations. In order to find out any potential markers of this disease, we characterized a collection of 101 scarlet fever isolates and compared it with pharyngitis isolates (n=202) (these not associated with scarlet fever). To achieve this, the superantigen gene profile was determined for all isolates, in addition to the typing methods already mentioned. The results showed that *ssa*, *speA* and *speC* were associated with scarlet fever and that scarlet fever isolates are less diverse than the pharyngitis isolates, indicating that a restricted number of lineages have a higher propensity to cause this presentation.

RESUMO

Palavras-chave: *Streptococcus pyogenes*, faringite, resistência aos macrólidos, variação no hospedeiro, escarlatina

Existem grandes variações geográficas e temporais na frequência de estirpes de *Streptococcus pyogenes* resistentes aos macrólidos, assim como na prevalência dos respectivos fenótipos de resistência. Apesar de uma diminuição na resistência aos macrólidos ter sido frequentemente associada a um baixo consumo deste antibiótico, foi já sugerido um papel importante para as flutuações naturais dos clones resistentes aos macrólidos na prevalência de estirpes resistentes e dos seus fenótipos.

Um estudo recente demonstrou a existência de heterogeneidade entre estirpes de estreptococos do grupo A (GAS) isoladas no mesmo doente, levantando a questão da validade de isolar e estudar uma só colónia a partir das placas primárias para a correcta determinação da resistência aos macrólidos. Para testar esta premissa, 321 estirpes de GAS, isoladas de 35 doentes do mesmo hospital, com um diagnóstico de faringo-amigdalite, foram estudadas de modo a detectar possíveis diferenças na susceptibilidade antimicrobiana e no tipo e subtipo *emm*. Nesta colecção, todas as estirpes isoladas do mesmo doente apresentaram o mesmo perfil de resistência aos antimicrobianos e o mesmo tipo e subtipo *emm*, indicando que a estratégia utilizada é adequada para uma correcta identificação da taxa de resistência aos macrólidos e para outros estudos epidemiológicos.

Ao longo desta dissertação, o estudo epidemiológico das estirpes de GAS resistentes aos macrólidos está dividida em 3 períodos - 1998 a 2003, 2004 a 2006 e 2007 a 2011. Em todos os períodos, a taxa de resistência aos macrólidos e a prevalência de cada um dos fenótipos e genótipos de resistência foram determinadas; a caracterização molecular da população resistente envolveu técnicas como a tipagem T, a tipagem *emm*, “pulsed field gel electrophoresis” (PFGE) e “multilocus sequence typing” (MLST).

No primeiro período, a resistência aos macrólidos foi elevada (27%), mas estável. No entanto, esta estabilidade não foi acompanhada por uma prevalência constante dos fenótipos de resistência. Em apenas 6 anos, assistiu-se a uma completa inversão na prevalência dos fenótipos de resistência. A caracterização molecular das 325 estirpes revelou uma população diversa e as linhagens genéticas identificadas em Portugal foram

também identificadas noutros países Europeus; as mais comuns eram definidas por T12-*emm*22-ST46, T28-*emm*28-ST52, T4-*emm*4-ST39, T12-*emm*12-ST36 e T1-*emm*1-ST28. Esta situação, muito pouco usual, motivou a continuação dos estudos de vigilância epidemiológica e os resultados da análise das estirpes isoladas entre 2004 e 2006 permitiram a detecção de uma tendência decrescente na resistência aos macrólidos, que começara em 1999. Era ainda evidente uma diversidade na composição clonal da população que incluía 156 estirpes, e, com pequenas excepções, foram identificadas as mesmas linhagens genéticas, com algumas diferenças na sua prevalência. Entre as linhagens mais frequentes estavam as definidas por T28-*emm*28-ST52, T4-*emm*4-ST39, T11-*emm*11-ST403 e T12-*emm*22-ST46. Para saber se esta tendência decrescente ainda se mantinha, a resistência aos macrólidos entre 2007 e 2011 foi determinada e a composição clonal da população foi estudada (n=139). Os resultados mostraram que a tendência decrescente da resistência aos macrólidos ainda se mantinha, atingindo-se em 2011 o valor mais baixo registado em Portugal (2%), e mais uma vez, as linhagens genéticas tinham sido já descritas em Portugal e noutros países, com algumas diferenças na sua prevalência. A linhagem genética definida por T11-*emm*11-ST403 era agora a mais prevalente, seguida por T12-*emm*22-ST46 e T28-*emm*28-ST52.

Uma das observações foi o facto de esta situação ter sido acompanhada por um elevado consumo de macrólidos, conferindo assim ênfase às flutuações dos clones na prevalência da resistência a este antibiótico.

O grupo relativamente restrito de linhagens genéticas com resistência aos macrólidos está bem disseminado, mas pouco se sabe acerca dos motivos para o seu sucesso ou da sua origem - aquisição de genes de resistência seguida de expansão local, ou simplesmente disseminação geográfica. Para tentar perceber se a população de GAS estava a reflectir a população geral de *S. pyogenes* isolados na faringe, uma colecção de 803 estirpes, representando 50% do total de estirpes isoladas em doentes com faringo-amigdalite em Portugal, entre 2000 e 2005, foi analisada por todos os métodos de tipagem acima mencionados. Esta comparação permitiu a identificação de tipos *emm* específicos e clones de PFGE associados com cada um dos fenótipos de resistência aos macrólidos. Entre os tipos *emm* encontrados nesta colecção, os tipos *emm*4, *emm*22 e *emm*28 foram associados com a resistência aos macrólidos, enquanto os tipos *emm*3, *emm*6, *emm*87 e *emm*89 foram associados com a susceptibilidade a este antibiótico. A utilidade do PFGE na

tipagem de GAS foi reforçada neste trabalho pois esta foi a única técnica de tipagem capaz de diferenciar estirpes resistentes e susceptíveis dentro do mesmo tipo *emm*. Foram exemplos disso grupos de estirpes dos tipos *emm*1 e *emm*4. Adicionalmente, ambas as populações mostraram diferenças na diversidade, indicando que a população de GAS resistentes aos macrólidos tem uma dinâmica própria, independente dos padrões de variação da população geral.

Durante muito tempo, a produção das exotoxinas SpeA e SpeC foi associada ao desenvolvimento de escarlatina, uma das apresentações das infecções causadas por *S. pyogenes*. No entanto, a literatura é contraditória e alguns estudos não detectaram esta associação. Para tentar encontrar potenciais marcadores desta infecção, caracterizámos uma colecção de 101 estirpes associadas a escarlatina e comparámos com 202 estirpes associadas a faringite (sem o diagnóstico de escarlatina). Para isto, foi determinado o perfil de superantigénios, para além de todos os métodos de tipagem já mencionados. Os resultados mostraram que os genes *ssa*, *speA* e *speC* estavam associados a escarlatina, mas foi também demonstrado que as estirpes associadas a escarlatina eram menos diversas que as estirpes associadas a faringite, o que sugere que existam linhagens de maior propensão para causar esta apresentação.

THESIS OUTLINE

The main purpose of the work presented in this thesis was the characterization of *Streptococcus pyogenes* associated with tonsillo-pharyngitis in Portugal, with a special focus on macrolide resistance. To achieve this, phenotypic and molecular typing methods were used among a large collection of isolates.

The study is presented in 6 chapters, organized as follows:

Chapter 1: General Introduction - This chapter provided a review of the main characteristics of this important pathogen - the infections it causes, its virulence factors, antimicrobial resistance, typing methods and epidemiology of macrolide resistance.

Chapter 2: Intrahost variation in infection - The heterogeneity among *S. pyogenes* isolates recovered from the same patient, in the course of pharyngitis is determined, in terms of antimicrobial resistance profiles and *emm* type.

Chapter 3: Epidemiological study of macrolide-resistant *S. pyogenes* isolates in Portugal (1998-2011) - In this chapter, the macrolide resistant GAS isolates recovered from tonsillo-pharyngitis in Portugal, between 1998 and 2011 are characterized. As explained in this chapter, this issue is divided into 4 subchapters corresponding to different time periods.

Chapter 4: Differences between macrolide resistant and susceptible *Streptococcus pyogenes*: importance of clonal properties in addition to antibiotic consumption - this chapter provides some clues about the origin of macrolide resistant clones in Portugal, by comparing a large collection of macrolide resistant and susceptible isolates.

Chapter 5: Scarlet fever is caused by a limited number of *Streptococcus pyogenes* lineages and is associated with the exotoxins *ssa*, *speA* and *spec* - in this chapter, the characterization of scarlet fever isolates is performed, in an attempt to identify the potential molecular markers for the development of scarlet fever.

Chapter 6: General Discussion - this chapter provides a discussion of the major findings obtained in this thesis, highlighting new perspectives for future work.

This thesis is based on the following papers:

C. Silva-Costa, M. Ramirez, J. Melo-Cristino, and the Portuguese Surveillance Group for the Study of Respiratory Pathogens. 2005. Rapid Inversion of the Prevalences of Macrolide Resistance Phenotypes Paralleled by a Diversification of T and *emm* Types among *Streptococcus pyogenes* in Portugal Antimicrob. Agents Chemother. 49:2109-2111.

C. Silva-Costa, M. Ramirez and J. Melo-Cristino. 2006. Identification of macrolide-resistant clones of *Streptococcus pyogenes* in Portugal. Clin. Microbiol. Infect. 12: 513–518.

C. Silva-Costa, F. R. Pinto, M. Ramirez, J. Melo-Cristino and The Portuguese Surveillance Group for the Study of Respiratory Pathogens. 2008. Decrease in macrolide resistance and clonal instability among *Streptococcus pyogenes* in Portugal. Clin. Microbiol. Infect. 14: 1152–1159.

C. Silva-Costa, A. Friães, M. Ramirez, J. Melo-Cristino, and the Portuguese Group for the Study of Streptococcal Infections. 2012. Differences between Macrolide-Resistant and -Susceptible *Streptococcus pyogenes*: Importance of Clonal Properties in Addition to Antibiotic Consumption. Antimicrob. Agents Chemother. 56:5661-5666.

C. Silva-Costa, J. Carriço, M. Ramirez and J. Melo-Cristino. 2013. Scarlet fever is caused by a limited number of *Streptococcus pyogenes* lineages and is associated with the exotoxin genes *ssa*, *speA* and *spec.* Ped. Infect. Dis. J., Accepted for publication

ABBREVIATIONS

ASO - anti-streptolysin O

AW - adjusted Wallace

CDC - Centers for Disease Control and Prevention

CI - confidence interval

CLSI - Clinical and Laboratory Standards Institute

$cMLS_B$- macrolides, lincosamides and streptogramin B (constitutive resistance phenotype)

DLV - double locus variant

DNA - deoxyribonucleic acid

erm - erythromycin ribosomal methylase

FCT - Fibronectin-binding, collagen-binding, T antigen

FDR - False discovery rate

GAS - Group A *Streptococcus*

$iMLS_B$- macrolides, lincosamides and streptogramin B (inducible resistance phenotype)

M - Macrolides (resistance phenotype)

mef - macrolide efflux

MIC - minimum inhibitory concentration

MLS_B - macrolides, lincosamides and streptogramin B (resistance phenotype)

MLST - multilocus sequence typing

mRNA- messenger ribonucleic acid

OR - odds ratio

PCR- polymerase chain reaction

PFGE - pulsed-field gel electrophoresis

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

SOF - serum opacity factor

Spe - streptococcal pyrogenic exotoxins

ST - sequence type

STSS - streptococcal toxic shock syndrome

tRNA - transfer ribonucleic acid

UPGMA - unweighted pair group method with arithmetic mean

W - Wallace

WGS - whole genome sequence

TABLE OF CONTENTS

ACNOWLEDGMENTS i

SUMMARY iii

RESUMO vii

THESIS OUTLINE xi

ABBREVIATIONS xiii

CHAPTER 1: GENERAL INTRODUCTION 1

1. *Streptococcus pyogenes* - general features 3

1.1 Identification 3

1.2 Virulence factors 4

1.3 Carriage 14

1.4 Infections 15

1.4.1 Suppurative infections 16

1.4.2 Post-infectious sequelae 20

1.5 Microbial diagnosis 21

1.6 Typing methods 22

1.7 Antimicrobial resistance and treatment of GAS infections 30

1.7.1 β-lactams 30

1.7.2 Tetracyclines 31

1.7.3 Macrolides and lincosamides 31

1.7.4 Streptogramins 32

1.7.5 Macrolide resistance mechanisms in GAS 31

1.7.6 Epidemiology of macrolide resistance 34

References 40

Aims of the Thesis 60

CHAPTER 2: INTRAHOST VARIATION IN INFECTION 63

Introduction 65

Materials and methods 67

Results 68

Discussion 70

References 73

CHAPTER 3

EPIDEMIOLOGICAL STUDY OF MACROLIDE RESISTANT *S. PYOGENES* ISOLATES IN PORTUGAL (1998-2011) 77

Brief introduction 79

CHAPTER 3.1

RAPID INVERSION OF THE PREVALENCE OF MACROLIDE RESISTANCE PHENOTYPES PARALLELED BY A DIVERSIFICATION OF T AND *emm* TYPES AMONG *STREPTOCOCCUS PYOGENES* IN PORTUGAL 83

CHAPTER 3.2

IDENTIFICATION OF MACROLIDE RESISTANT CLONES OF *STREPTOCOCCUS PYOGENES* IN PORTUGAL 93

CHAPTER 3.3:

DECREASE IN MACROLIDE RESISTANCE AND CLONAL INSTABILITY AMONG *STREPTOCOCCUS PYOGENES* IN PORTUGAL 111

CHAPTER 3.4:

MACROLIDE RESISTANCE IN *STREPTOCOCCUS PYOGENES* IN PORTUGAL (2007-2011): DIMINISHING RESISTANCE AND CLONAL INSTABILITY, IN SPITE OF HIGH MACROLIDE CONSUMPTION 133

CHAPTER 4:

DIFFERENCES BETWEEN MACROLIDE RESISTANT AND SUSCEPTIBLE *STREPTOCOCCUS PYOGENES*: IMPORTANCE OF CLONAL PROPERTIES IN ADDITION TO ANTIBIOTIC CONSUMPTION 157

CHAPTER 5:

SCARLET FEVER IS CAUSED BY A LIMITED NUMBER OF *STREPTOCOCCUS PYOGENES* LINEAGES AND IS ASSOCIATED WITH THE EXOTOXIN GENES *ssa*, *speA* and *speC* 179

CHAPTER 6: GENERAL DISCUSSION 201

Intrahost variation in infection 203

Epidemiology of macrolide resistant GAS in Portugal 204

Erythromycin resistant and susceptible GAS 208

Scarlet fever GAS 209

Concluding remarks and future perspectives 210

References 213

CHAPTER 1

GENERAL INTRODUCTION

1. *Streptococcus pyogenes* - general features

The presence of streptococci in patients with skin infections was first demonstrated in 1874 by Billroth and in 1879, Pasteur detected the presence of these microorganisms in the blood of a patient with puerperal sepsis. In 1883, Fehleisen established the importance of these chain forming organisms in human infections and the designation of *Streptococcus pyogenes* was finally applied in 1884 by Rosenbach [30].

Streptococcus pyogenes is a Gram positive bacterium, identified as Lancefield's group A streptococci (GAS) in 1933 [117]. Individual cells have spherical shape, with 0.5-1 μm size, are nonmotile and normally arranged in pairs or straight chains. It is a facultative anaerobe microorganism, catalase and oxidase negative [147]. The Lancefield classification scheme distinguished these streptococci based on their group A carbohydrate, composed of N-acetylglucosamine linked to a rhamnose polymer backbone [117]. On blood agar plates, colonies form large zones of beta-hemolysis, that result from the production of streptolysin-O that mediates the complete lysis of erythrocytes [153] (see Figure 1.1).

1.1. Identification

Streptococcus pyogenes identification starts with the observation of beta-hemolytic colonies, when grown in 5% blood agar plates (Figure 1.1). Presumptive identification can be made using the bacitracin susceptibility test [70], but it is not a very reliable test, since bacitracin resistant *S. pyogenes* isolates were already described [132, 141, 160].

FIGURE 1.1: β-hemolysis produced by *S. pyogenes* colonies on blood agar plates

Based on the serological classification developed by Rebecca Lancefield in 1933 [117], there are commercially available kits for the presumptive species identification of beta-hemolytic streptococci that involve the enzymatic extraction of the carbohydrate and its agglutination reaction with specific sera. A positive reaction can also be seen between the group A serum and other beta-hemolytic streptococcal species, such as *S. dysgalactiae* subsp. *equisimilis* and *S. anginosus* group, but these are more rarely associated with human disease [70].

Other test for the identification of *S. pyogenes* include pyrrolidonylarylamidase activity, since only this species among streptococci produces the enzyme [70].

1.2. Virulence factors

Streptococcus pyogenes expresses multiple virulence factors, which are involved in several aspects of host-pathogen interactions.

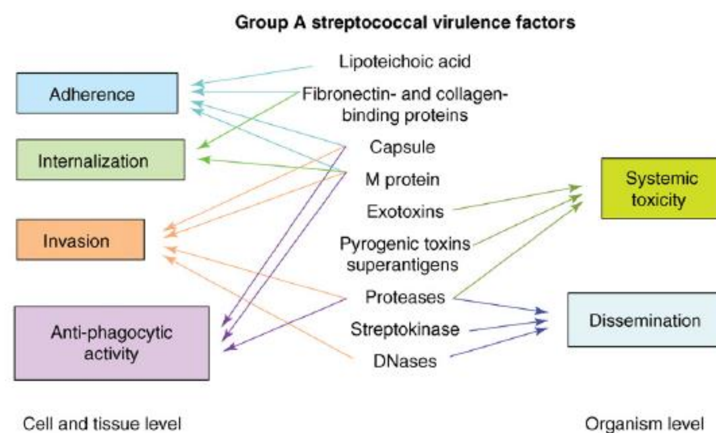

FIGURE 1.2: GAS virulence factors interact with the host at many levels. At cell and tissue level, the virulence factors contribute to the pathogenicity of GAS mediating adherence to host cells, promoting internalization and invasion and circumventing phagocytosis. At the organism level, these factors are involved in the dissemination throughout the host and induction of systemic toxicity. Many of the known GAS virulence factors are involved in more than one stage of infection. Reproduced from Tart *et al*. [203].

Initial contact between GAS and human epithelial cells is a very important step because without strong adherence, the microorganism would be removed by mucous and

salivary fluid flow and through the exfoliation of the epithelium [57]. Moreover, it has been suggested that the invasion of deeper tissues from the mucosa or skin may be facilitated by specific adhesions mechanisms [57]. The adherence process may also activate the immune system by inducing local cytokine production and inflammatory responses [54, 212].

The adherence of this pathogen to the host cells, the invasion of host tissues, the interaction with the immune system and the production of several enzymes and toxins are important to the establishment of GAS disease in the human host and multiple virulence factors are involved in all stages of infection.

A brief description of the major virulence factors will be addressed in the next section.

Capsular Polysaccharide

Based on the morphology of the colonies of GAS when grown on solid media, Rebecca Lancefield distinguished three types of hemolytic streptococci – mucoid (translucent, liquid appearance), matte (irregular, collapsed appearance) and glossy (compact, opaque) colonies and established an association between virulence and the appearance of the colonies. Mucoid or matte colonies were associated with potentially virulent isolates for mice, while glossy colonies were formed by less virulent isolates. Furthermore, isolates producing mucoid or matte colonies were shown to produce higher levels of M protein relatively to the glossy colonies isolates [120, 208]. The M protein received its designation because of this association with mucoid or matte colonies [219]. In 1959, Wilson demonstrated that the mucoid or matte morphology of the colonies was due to the production of a capsular polysaccharide, instead of the M protein [219]. However, encapsulated strains can be difficult to identify after subculture in the microbiology laboratory, since the expression of the capsule can be lost in some conditions, such as the growth in artificial media [196].

The GAS capsular polysaccharide is composed of hyaluronic acid, a high-molecular weight linear polymer containing D-glucoronic acid and N-acetylglucosamine. The similarity between capsular hyaluronate and the one found in human connective tissue renders this virulence factor a poor immunogen [28]. The importance of the capsule as a

major virulence factor of *S. pyogenes* comes from the frequently reported association between invasive infections and rheumatic fever and mucoid strains [197].

Capsule production is considerably variable among GAS isolates. The capsule acts as a virulence factor by preventing phagocytosis, as already demonstrated by studies with acapsular mutants [217]. Furthermore, in highly encapsulated isolates, the treatment with hyaluronidase rendered them more susceptible to phagocytosis, supporting a protective role of the capsule [174]. The mechanism used by the capsule in preventing phagocytosis is the inhibition of complement activity by blocking the access of neutrophils to the opsonic complement components bound to the bacterial surface, rather than inhibiting C3b deposition [216].

The hyaluronic acid capsule of GAS has been shown to enhance virulence in skin and soft tissue infections [5, 181], as well as in systemic infections [217]. This could be due to its interference with the immune system but the capsule was also shown to influence the attachment to the epithelium of the pharyngeal mucosa and the skin, either by modulating the interactions of the M protein and other adhesins or by direct binding itself to CD44 on human keratinocytes [180]. It was already demonstrated that the binding of the GAS hyaluronic acid capsule to CD44 on epithelial cells triggers cytoskeletal rearrangements that open intracellular junctions, in a serotype-specific manner, facilitating paracellular translocation of the microorganism which can be used as a possible mechanism of tissue invasion [58, 199].

M protein

In 1927, Rebecca Lancefield identified the M protein as a “type-specific substance”, demonstrating its ability to elicit an immune response [118], that is protective against re-infection with the same M serotype [119]. On the bacterial surface, the proteins are arranged in hair-like structures that protrude away from the surface [154] (Figure 1.3)

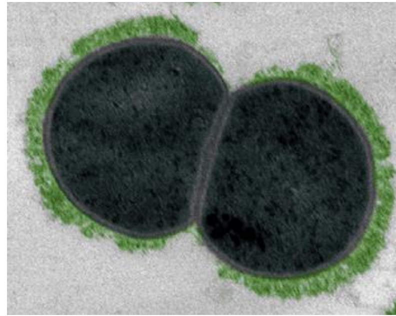

FIGURE 1.3: Transmission electron micrograph of a thin sectioned *S. pyogenes* bacterium, with the hair-like structure colored in green. Reproduced from Oehmcke *et al*. [154]

The M protein is formed by two polypeptide chains that form an alpha-helical coiled-coil arrangement, anchored in the cell membrane and spanning the cell wall [28] (Figure 1.4). Typically, the polypeptide chains consist of four blocks (labeled A-D), which differ in size and aminoacid sequence (Figure 1.4). The structure of the C-terminal has an LPXTG motif, a signal required for the correct anchoring of the protein to the cell wall and this region is highly conserved among GAS strains [81]. The amino terminal region, that extends to the exterior is a highly variable region and constitutes the serotype specificity of different GAS isolates [28].

The repeats located in the conserved region of the protein are involved in the interaction with the immune system [103], and the fibrinogen binding domain of many M proteins is contained in the variable region [169].

The size of the M proteins is variable among *S. pyogenes* isolates, even among strains of the same serotype [80, 104]. This is thought to be generated by homologous recombination between intragenic repeats, resulting in the addition or deletion of repeats [104]. A recent study found that the whole surface-exposed sequence of the M protein shows significant size variation, ranging from 200 to 435 aa among a large collection of isolates, representing multiple M types [139]. This size variation is related to the variable number of repeats each M protein harbors. Some proteins lack “A” and “B” repeats entirely and sometimes the “B” repeats are present as a single repeat (Figure 1.4) [139]. As different regions of the M protein interact with different host proteins and components of the immune system, it is possible that changes in the size of the M protein can give individual bacteria a survival advantage, but further studies of the impact of the size of the M protein in the virulence potential and clinical manifestations are still needed [139].

The M protein is encoded by the *emm* gene and analysis of the presence and arrangement of the *emm* family genes in the bacterial chromosome allowed the distinction of 5 dominant *emm* patterns (patterns A-E), with few exceptions. It was also possible to establish significant associations between some of these patterns and different diseases: pharyngeal isolates were associated with *emm* patterns A, B or C, and pattern D is associated with skin isolates. Among isolates presenting pattern E, no significant association could be established with any of the sites of infection (skin or throat). These results provided the genetic basis for the early recognized concepts of “throat” and “skin” strains, leading to the idea that strains with the A-C genotype tend to show a strong preference for throat infections (“throat-specialists”), *emm* pattern D strains have a tendency to cause impetigo (“skin-specialists”) and *emm* pattern E strains infect both tissues (“generalists”) [19].

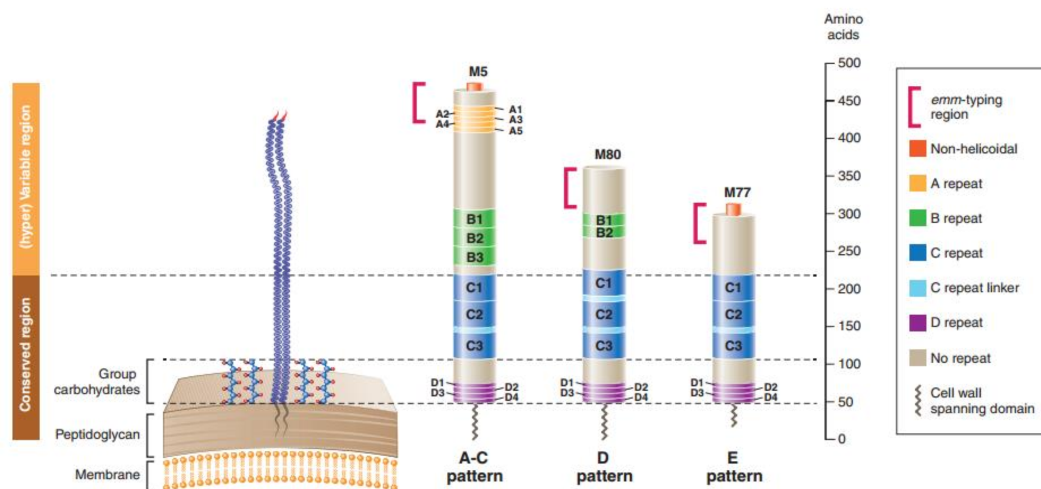

FIGURE 1.4 Three representative M proteins model. Patterns A-C *emm* types represent the longest M proteins. The “A” repeats are absent from the vast majority of M proteins belonging to the pattern D and E. The “B” repeats are present in most A-C and D *emm* types, but absent from most of the pattern E *emm* types. Reproduced from McMillan *et al*. [139]

M proteins can be classified into two groups (class I and class II) based on the presence or absence of a conserved antigenic domain within the surface-exposed portion. The class I molecules show immunoreactivity with C repeat region specific antibodies, while the class II molecules do not react with these antibodies [21]. This classification is further supported by differences in the biological properties of both classes. GAS isolates carrying class II M proteins are usually producers of an apoproteinase called the serum opacity factor (SOF) that causes the aggregation of the high-density lipoprotein and

opacification of serum [179]. In contrast, isolates carrying class I molecules fail to produce SOF [57]. Moreover, class I M protein serotypes are associated with rheumatic fever and class II molecules are associated with acute glomerulonephritis [20, 21, 154].

The M proteins are believed to be the primary antigens of GAS and anti-streptococcal antibodies also reacting against a variety of human proteins have been described, such as cardiac myosin, tropomyosin, vimentin, laminin, keratin and several valvular proteins. This molecular mimicry of the M protein is particularly important in the induction of autoimmune reactions in rheumatic fever, a non-suppurative sequela caused by *S. pyogenes* infections [154].

The M protein was also recognized as an important adhesin [67], through the interaction with receptors in the host cells, such as fibronectin, membrane cofactor protein (CD46) and glycosaminoglycans, an interaction that depends on the M type and the target host cell [57, 87, 154]. The M protein has antiphagocytic properties, avoiding complement activation by binding at least one of the following complement factors - factor H, factor H like-1 (FHL-1), C4-binding proteins (C4BP) and CD46 complement regulatory protein. The interaction of the M protein with factor H or FHL-1 seems to occur in different regions of the hypervariable portion of the protein and disrupts the alternative complement pathway by inhibiting the deposition of C3b and reducing phagocytosis by polimorphonuclear leucocytes [27, 57]. Binding of C4BP to the hypervariable region of the M protein (an inhibitor of the classical complement pathway) has been shown to confer phagocytosis resistance. The M protein can also bind fibrinogen, decreasing the deposition of C3b in the bacterial surface, activating the alternative complement pathway or blocking the classical pathway [154].

Although considered an extracellular pathogen, GAS internalization into cultured human epithelial cells has already been demonstrated [121, 143] and the binding of M protein to host cell receptors seems to contribute to this uptake [56].

T protein

The T protein is a tripsine-resistant surface protein of GAS, and its variability constitutes the basis of a typing system [92]. For a long time, the T protein was regarded solely as an epidemiological marker, with an unclear role in GAS virulence. However, it

was recently shown that *S. pyogenes* harbors, in its surface, pilus-like structures, composed of members of a family of extracellular matrix binding proteins (ECM-binding proteins). It was also demonstrated that the Lancefield T6 antigen forms the backbone of one of the pili and the backbone of three other pili was recognized by three different sera from the T-typing system, suggesting that the diversity of these pilus-like structures are the basis for this typing system [145].

GAS pili are encoded in a region termed FCT (Fibronectin-binding, Collagen-binding T antigen) region, and nine FCT variants have been identified so far [72]. Because they are members of a family of ECM- binding proteins, their contribution to virulence is believed to be related to adhesion and invasion of epithelial cells, but a role in biofilm formation was also suggested [13, 134]. Additionally, recombinant pilus proteins showed the capacity to induce protective immunity in a mouse model of GAS infection and invasive disease [145], reinforcing this importance as a virulence factor.

Fibronectin-binding proteins

The adherence and invasion of host cells by *S. pyogenes* are also mediated by fibronectin binding proteins. There are currently 11 fribronectin-binding proteins described in GAS, including Protein F1 (PrtF1)/SfbI, Protein F2 (PrtF2)/PFBP, SOF (serum opacity factor)/SfbII, FbaA and FbaB (fribronectin binding proteins A and B), the M1 protein and Scl1 (streptococcal collagen-like protein 1). The distribution of fribronectin binding proteins among GAS isolates seems to be related to the M type, while the correlation with clinical manifestations is not so clear [158, 220]. Moreover, it was suggested that the presence of some of these proteins could be an important epidemiological marker, as demonstrated for the higher prevalence of the *prtF1* and/or the *prtF2* genes among macrolide resistant GAS, that was suggested to represent protection of the action of antimicrobial treatment and of the host immune response [6, 97]. Some of the fribronectin binding proteins of GAS also promote evasion of phagocytosis by the inhibition of complement activity [220].

In the human host, GAS encounters various barriers, including a vigorous innate immune response. A variety of virulence determinants allow *S. pyogenes* to survive in this

new environment, avoiding recognition and phagocytosis and establishing long-term survival within the host. Besides the already mentioned M protein, the hyaluronic acid capsule, and some fribronectin-binding proteins, *S. pyogenes* produces a variety of extracellular products important for virulence by interacting with the host immune system. Among these are streptococcal pyrogenic exotoxins, streptodornases (DNases), streptolysin O and streptolysin S, interleukine-8-protease (SpyCEP), streptococcal inhibitor of complement (Sic), C5a peptidase and streptokinase [52].

Streptococcal pyrogenic exotoxins

S. pyogenes produces many virulence factors and among them are the secreted pyrogenic exotoxins (Spe), which act as superantigens. These superantigens interact with the host major histocompatibility complex (MHC) class II molecules and with the variable region of the T-cell receptor β-chain. Unlike conventional antigens, this interaction occurs without the involvement of antigen-presenting cells. Moreover superantigens can interact with large numbers of T cells that share particular sequences within the variable region of the β chain of the T cell receptor, resulting in an extensive immune activation and significant release of pro-inflammatory cytokines, leading to the shock and organ damage seen in some severe GAS infections [113].

There are 11 superantigens described so far in *S. pyogenes*: some of these superantigens are chromosomally encoded (SpeG, SpeJ and SMEZ) and others are encoded on temperate bacteriophages (SpeA, SpeC, SpeH, SpeI, SpeK, SpeL, SpeM and SSA). The chromosomally encoded SpeB and SpeF are actually a cysteine protease and a DNase, respectively, instead of superantigens, in contrast to what was originally described [88, 109]. SpeB is one of the best-studied virulence factors of GAS. It promotes evasion from the immune system, degrading a wide range of host and GAS proteins, promoting escape of recognition and phagocytosis and facilitating tissue invasion and spread [150].

Streptodornases (DNases)

NETs (neutrophil extracellular traps), secreted by host neutrophils in response to chemotactic signals are composed of DNA, histones, granule proteases and antimicrobial peptides. Their role is to facilitate the entrapment and clearance of bacteria in the initial site of infection, independent of phagocytic uptake. To overcome NETs, *S. pyogenes* produce one or more streptodornases (DNases). An example is the phage-encoded DNase (sda1), produced by *S. pyogenes* T1M1, frequently associated with invasive infections, with a demonstrated role in NET escape and virulence [40].

Streptolysin O and Streptolysin S

Streptolysin S (SLS) is an oxygen-stable, non-immunogenic hemolysin, with a broad cytolytic spectrum, being involved in phagocytic clearance and cell cytotoxity. The expression of SLS promotes virulence in animal models of invasive infection and contributes to GAS translocation across epithelial barriers [60, 199].

Streptolysin O (SLO) is an oxygen-sensitive hemolysin that binds to cholesterol in eucaryotic cell membranes, where it oligomerizes to produce large transmembrane pores, leading to cell lysis. It is immunogenic and increased titers of anti-SLO (ASO) are good indicators of streptococcal disease [50]. SLO facilitates the escape of GAS from the endosome-lysosome pathway following invasion of host cells [96], and contributes directly to virulence in animal models of systemic infection [207].

C5a peptidase

C5a peptidase is a proteolytic enzyme anchored to the cell wall of *S. pyogenes*, encoded by the *scpA* gene, which interferes with the immune system by inhibiting the recruitment of phagocytic cells to the site of infection, through the cleavage of C5a at its polimorphonuclear-binding site [57]. As a result, the infiltration of phagocytes is delayed and the clearance of bacteria is retarded, allowing the establishment of the infection. C5a peptidase is capable of inducing a strong immune response, with a good correlation to the

response of anti-streptolysin O (ASO) and anti-DNase B, which are considered indicators of GAS infection [187].

Interleukin-8 protease (SpyCEP)

SpyCEP (ScpC) is a cell wall anchored proteinase which degrades interleukin-8 (IL-8), a chemokine that mediates neutrophils transmigration and activation, allowing bacterial spread and development of systemic infection [102]. However, the ability to degrade IL-8 differs dramatically among clinical isolates and it was already suggested that the contribution of this virulence factor to disease outcome depends on the site of infection and on host environment [190].

Streptococcal inhibitor of complement (Sic)

Sic is a highly polymorphic secreted protein, produced by very few strains of GAS, mostly by M1 isolates. Initially it was thought that its role in GAS virulence was related solely to an interference with the formation of the membrane attack complex (MAC), but it was recently demonstrated that it is, in fact, a polyfunctional inhibitor of the innate immune response, inhibiting the activity of a wide range of antimicrobial peptides and proteins [77].

Streptokinases

One of the proteins secreted by GAS is the streptokinase with the ability to convert plasminogen to plasmin [154]. The protease activity of plasmin is important in bacterial colonization, degrading blood clots and extracellular matrix components (ECM) and activating metalloproteinases. GAS can interact with human plasminogen directly through specific surface proteins or indirectly through fribronectin-binding proteins and the presence of plasminogen at the infection site, leading to increases in the local levels of plasmin. This was proposed to have an important role in GAS pathogenesis, demonstrated by increased virulence in mouse skin models and enhanced adherence and invasion in pharyngeal cells [154, 159].

Global virulence regulators

The expression of virulence factors is controlled by transcriptional regulators, that respond to environmental signals present in the various niches which *S. pyogenes* encounters in the human host. With the increasing availability of complete genome sequences, a reasonable number of response regulators and two component signal transduction systems were described, summarized in a review from Kreikemeyer and collaborators [114]. Among the response regulators are Mga (regulating genes encoding adhesins and proteins involved in tissue invasion, such as *emm*, *sof*, *scpA* and *sic*, among others), RofA-like protein (RALP) family, regulating genes encoding fribronectin binding proteins, hemolysins, proteases, superantigens and other virulence regulators (Mga) and Rgg/RopB, that regulates the expression of SpeB, exerting a negative effect on *mga* transcription and influencing several two component system networks [114].

Signal transduction through two component systems include the Ihk/Irr, with an essential role in GAS evasion of polimorphonuclear leucocyte (PMN) - mediated killing being highly expressed during acute pharyngitis and FasBCAX, involved in the down-regulation of genes encoding adhesins and induction of genes encoding secreted streptolysin S (*sagA*) and streptokinase (*ska*). These two systems display homology with systems from S*taphylococcus aureus* and other streptococcal species. The best-characterized two component system of GAS is CsrRS (csr standing for capsule synthesis regulator) also called CovRS (cov standing for control of virulence genes). This system was already shown to regulate capsule gene transcription and the expression of important virulence factors, such as streptolysin S, streptokinase, streptodornases and the cysteine protease SpeB [114].

1.3. Carriage

The ecological niche of *Streptococcus pyogenes* is quite narrow; their only known host are the humans. The primary sites for *S. pyogenes* colonization involve the nasal and oropharyngeal mucosal epithelium in the upper respiratory tract and the superficial layers

of the epidermis [18]. Since GAS has no environmental reservoir, direct transmission via respiratory droplets or skin contact is essential for its survival and dissemination [22].

Some individuals may harbor GAS continuously for months or years without symptoms of infection [107]. The prevalence of streptococcal carriers is a controversial matter, since many differences in their number were found, depending on the study population and the methods used [163]. A meta-analysis of the prevalence of streptococcal carriage in children revealed that among children younger than 5 years, the pooled prevalence of GAS carriage was 4%, ranging from 2% to 17%, lower than the carriage in children of all ages (12%, ranging from 3% to 26%) [186]. Chronic carriers can represent a clinical challenge, because even when the pharyngitis has a viral cause, GAS can still be detected by the culture of a throat swab, potentially confounding the ethiological diagnosis. Streptococcal carriage may not be effectively abolished by antimicrobial therapy [171] and there are several reasons for this, such as the internalization of the bacteria in the tonsillar epithelium cells [121, 149], the production of beta-lactamases by other microorganisms present in the oropharynx or the absence of a normal flora, capable of inhibiting GAS [39].

In Portugal, a study conducted between 2000 and 2006 in the Lisbon area that included children and adolescents attending day care centers and schools and close contacts, reported a rate of oropharyngeal carriage of *Streptococcus pyogenes* of 11% in children and 3.3% in adults [164].

1.4. Infections

Streptococcus pyogenes causes a broad spectrum of diseases, ranging from uncomplicated infections in the upper respiratory tract and skin to severe, life-threatening infections, as well as post-infectious complications [57].

The global burden of GAS diseases is difficult to estimate. Some of the infections caused by *S. pyogenes* are very common in low-end economic settings and developing countries, where usually there are no systems to accurately estimate the disease burden. Given this limitation, any attempt to estimate the global prevalence of GAS infections will lack important information [41]. Still, with the available data, it was estimated that there are at least 517 000 deaths each year due to severe GAS diseases and the greatest burden is

due to rheumatic heart disease, with a prevalence of at least 15.6 million cases, with 282000 new cases and 163000 deaths each year. Additionally, there are more than 111 million cases of pyoderma and over 616 million cases of pharyngitis worldwide each year [41].

1.4.1 Supurative infections

Pharingytis

Pharyngitis is one of the most common infections for which patients seek medical advice [50]. A variety of bacterial and viral agents can cause pharyngitis in the immunocompetent host. The most frequent include viral agents, of which rhinovirus, coronavirus and adenovirus are the most important [95]. Among bacterial agents, *Streptococcus pyogenes* is, by far, the most frequent pathogen isolated from pharyngitis, being responsible for 5-15% of pharyngitis cases in adults and 20-30% in children, most commonly in children aged 5-15 years [218]. However, under crowded conditions, such as schools, day care centers and military facilities, all ages are vulnerable to the spread of the organism [57]. Exudative pharyngitis due to streptococcal infection among children younger than 3 years of age is rare [218]. Other streptococci can also be responsible for pharyngitis, although to a lesser extent, such as group C and group G streptococci, presenting clinical features that are very similar to pharyngitis caused by *S. pyogenes* [95]. Other less common bacterial agents include *Neisseria gonorrheae*, *Arcanobacterium haemolyticum*, some species of *Corynebacterium*, and Chlamydial and Mycoplasmal species [95].

The characteristic symptoms of streptococcal pharyngitis include a sudden onset of sore throat, pain on swallowing, fever, headache, abdominal pain and nausea and vomiting. Some clinical signs, such as tonsillopharyngeal exudate and erythema, soft-palate petechiae, beefy red and swollen uvula, anterior cervical lymphadenitis and scarlatiniform rash can also be present [26]. In the case of viral pharyngitis, respiratory signs and symptoms such as rhinorrhea, nasal congestion and cough are also present, although there is some evidence that these signs in patients with GAS pharyngitis may be more common than it was previously thought [135].

Scarlet fever

Scarlet fever, also known as scarlatina, is usually associated with throat infections, although it can also occur due to infections at other sites and is characterized by skin rash, "strawberry tongue" and sore throat [57]. It is considered a toxin-mediated disease, so that the streptococcal superantigens have historically been referred to as erythrogenic toxins or scarlet fever toxins. The characteristic scarlet fever rash is believed to be due to a hypersensitivity reaction that results from superantigen activity [137], mainly of SpeA, although it has been suggested that in the absence of this pyrogenic exotoxin, SpeC could also cause identical symptoms [221].

In the 19th and 20th centuries, it was a significant cause of childhood morbidity and mortality [146], and although scarlet fever outbreaks still occur, it is currently a relatively rare disease [45, 69, 116, 155, 209].

Skin and soft tissue infections

S. pyogenes is responsible for a variety of clinical conditions involving the skin and soft tissue. Localized streptococcal purulent infections of the skin are usually referred as GAS pyoderma, but generally this term is used mostly to refer to streptococcal impetigo and ecthyma [135].

Impetigo is a highly contagious infection of the skin, although superficial and self-limiting, which affects primarily exposed areas (face, arms and legs). At the skin, GAS can survive and replicate, reaching the subcutaneous tissues through small breaks in the skin, causing a strong inflammatory response resulting in the pyoderma purulent lesions. Systemic signs of infection are uncommon, but the spread of the infection by scratching is usual. GAS ecthyma occurs when lesions extend more deeply into the dermis and produce shallow ulcers [29, 135].

Erysipela is an acute inflammation of the skin that involves the skin and superficial lymphatic vessels. Clinically, an elevated erythematous lesion is present, and fever and other systemic signs of disease are common. Cellulitis is an infection that extends more deeply into subcutaneous tissues, with edema, redness, warmth and induration of the skin and/or soft tissue. As is the case with erysipelas, the presence of cellulitis may be

associated with systemic signs of infection and fever [29, 135]. Other species can be responsible for impetigo, erysipelas and cellulitis. *Staphylococcus aureus* is frequently recovered from impetigo lesions, alone or in combination with *S. pyogenes*, and some cases of cellulitis are caused by *S. aureus,* which has implications for empiric therapy, since the treatment of such infections requires the use of antimicrobial agents active against both species. Staphylococci are rarely responsible for erysipelas, but groups C, G and B streptococci can be responsible for this infection, although the majority of cases are caused by GAS [29].

Necrotizing fasciitis

Necrotizing fasciitis is a deep infection of subcutaneous tissue that results in the progressive destruction of fascia and fat. This infection is commonly associated with GAS bacteremia and the concomitant development of shock and organ failure, often in patients with streptococcal toxic shock syndrome, which will be discussed below [29]. Patients often have some prior history of trauma, such as an insect bite, scratch or abrasion and most of them have pre-existing conditions that render them more susceptible to this infection, such as varicella or specific conditions that result in immunosuppression such as diabetes mellitus, advanced age, chronic renal failure, peripheral vascular disease and drug misuse, although it also occurs in young and previously healthy individuals [29, 101].

The successful management of necrotizing fasciitis is dependent on a rapid diagnosis to allow for an adequate therapeutic response, requiring early and aggressive surgical debridement of the site of infection and antimicrobial therapy [135].

Streptococcal toxic shock syndrome

Streptococcal toxic shock syndrome (STSS) is characterized by a sudden onset of shock and organ failure and the presence of a culture positive for GAS. Cases have been observed more frequently in patients with risk factors, like those with chronic underlying conditions, such as diabetes mellitus, chronic cardiac or pulmonary diseases, immune-deficiency syndromes and children with varicella [135]. However, STSS can sporadically be present in previously healthy persons as primary peritonitis, with septic shock and

multiple organ failure emerging in just a few hours, with some cases occurring in women of reproductive age, suggesting an association with the female genital tract [206]. In fact, a portal of entry from the skin or vaginal mucosa was observed in nearly 60% of the patients; in other cases, a transient bacteremia originating in the pharynx could be the cause of the condition [29, 206]. Recent data from European countries revealed an incidence of STSS of 13% in streptococcal infections from any source, which increased dramatically to 50% in patients with necrotizing fasciitis, with a 7-day mortality of 44% [115].

STSS represents the most fulminant expression of disease caused by toxin-producing strains of *S. pyogenes*. It results from the ability of bacterial toxins to act as superantigens, triggering excessive and non-conventional T-cell activation, with concomitant activation of other cell types and stimulating massive cytokine production that ultimately causes tissue damage, disseminated intravascular coagulation and organ dysfunction [122].

Therapy with broad spectrum antibiotic should be instituted until the presence of GAS has been confirmed, with coverage for streptococci and *S. aureus*, and an association of clindamycin and a beta-lactam antibiotic is recommended to achieve a more effective treatment of GAS invasive disease. Penicillin is relatively ineffective in the treatment of soft-tissue infections given the high concentration of organisms with a slow rate of replication (the Eagle effect), being less susceptible to the inhibition of cell wall synthesis [29]. Clindamycin, with an effect on protein synthesis, has been shown to decrease the production of M protein and SpeA and is thought to lead to a better outcome due to reduction of the production of proteins interfering with the immune system [135, 193].

Interestingly, not all patients colonized or infected with a toxin-producer strain of *S. pyogenes* develop STSS, suggesting a role for the interaction between specific properties of the pathogen and of the host immune system in the development of this syndrome. Low levels of protective antibodies may contribute to the susceptibility of the host to invasive streptococcal infection [8] and an association between human leucocyte antigen class II haplotype and susceptibility to superantigen toxicity was already demonstrated [128].

1.4.2. Post-infectious sequelae

Rheumatic fever

Rheumatic fever is a delayed consequence of group A streptococcal pharyngitis. The major signs of the disease are polyarthritis, carditis, chorea and less frequently subcutaneous nodules and erythema marginatum, with minor signs of systemic inflammation potentially also evident [195]. It is an autoimmune disease that results from the production of autoreactive antibodies and T cells shown to cross-react with components of the group A streptococcus and host tissues. Streptococcal components include the M protein, which has epitopes that cross-react with cardiac and other host tissues [195].

It is now considered a rare disease, and its prevalence reflects, in part, the adequacy of preventive medical care. The site of the antecedent infection to rheumatic fever is pharyngeal, but not all pharyngitis caused by GAS progress to rheumatic fever [195]. Some studies have already shown that rheumatogenic strains express potentially some M types, with an association of some rheumatic fever epidemics to such M types, including M5, 3, 6, 14, 18, 9 and 24 [195]. Although some rheumatic fever outbreaks were reported in the 1980s, a decrease in the incidence of this disease in the last 50 years was already associated with the replacement of rheumatogenic types by non-rheumatogenic types in cases of acute pharyngitis in the USA [189].

Although it is believed that there is some genetic susceptibility to the disease, the association of rheumatic fever to specific host factors, like the human haplotypes is still not clear [197].

Glomerulonephritis

The clinical manifestations of glomerulonephritis, a nonsuppurative sequela of GAS infections include discolored or coffee-colored urine due to hematuria, edema of the face and extremities and circulatory congestion due to renal impairment. Unlike rheumatic fever, both pharyngeal and skin infections can lead to glomerulonephritis.

Like in the case of rheumatic fever, the nephrotoxicity of GAS seems to be related to specific M types, although not all strains of the same M type are nephritogenic. The

predominant M protein types associated with skin infections and pyoderma are M2, 49, 42, 55, 56, 57 and 60 while M types 1, 4, 12 and 25 are associated with throat infections and glomerulonephritis, although M types 12 and 1 are sometimes found in association with skin infections too [57, 173].

The development of poststreptococcal glomerulonephritis is related to an immunological response that seems to involve immune complexes with streptococcal antigenic components and subsequent glomerular deposition along with complement activation and molecular mimicry between streptococcal and renal components, leading to an autoimmune response [1]. The streptococcal antigens involved in the pathogenesis of the disease include the M protein, a streptococcal plasmin-binding protein (NAPlr) and SpeB [1, 57].

Previous studies have already reported associations between the incidence of glomerulonephritis and the presence of host susceptibility factors, including the HLA haplotypes [1].

1.5 Microbiological diagnosis

Clinical features of GAS can be nonspecific and do not discriminate between GAS and viral pharyngitis although particular signs are more typical viral features like rhinorrhea, cough, oral ulcers, and hoarseness [188]. Recommendations for the diagnosis of GAS include swabbing the throat and testing with a rapid antigen detection test (RADT), which detects the presence of group A streptococcal carbohydrate on a throat swab or culture [188]. Given the high incidence of pharyngitis in children and adolescents, it is also recommended that in case of a negative RADT, a culture should also be performed [188], because some RADTs appear to be significantly less sensitive than culture [31]. Culture of the throat swab in blood agar plates (BAP) was first described in 1954 by Breese and Disney [36] and remains the standard for the establishment of the presence of GAS in the upper respiratory tract [31], with the additional advantage of isolating the microorganism for epidemiological and antimicrobial resistance studies.

In a positive throat culture, *S. pyogenes* appear as beta-hemolytic colonies among other normal microbiota, which consists of species from the genera *Streptococcus*,

Haemophilus and *Neiseria* as well as *Staphylococcus* and other bacteria, although less frequently [126]. Some strategies have been described to achieve a better recovery of group A streptococci such as growing the microorganism under anaerobic conditions, improving in this way the activity of streptolysin O, and the incorporation of antibiotics such as sufamethoxazole-trimethoprim (SXT) in BAP in order to inhibit the growth of some of the normal microbiota of the upper respiratory tract [57, 142]. However, these approaches have to be used carefully, because *in vitro* resistance of *S. pyogenes* to SXT, initially believed to be common, seems to be dependent on the composition of the medium used [35].

1.6 Typing methods

There are several typing methods available for epidemiological studies of GAS, including phenotypic and molecular methods. The phenotypic methods, such as determination of antimicrobial susceptibility patterns and resistance phenotypes, phage typing or T typing, present the great advantage of being simple, rapid and effective in the identification of isolates recovered during an outbreak, but showed to be inadequate for evolutionary studies. Besides their low discriminatory power, the number of GAS isolates non-typeable with these methods (particularly in the case of T typing), reinforced the need to complement these methods with molecular typing methods [75, 156].

M- and T-typing

Apart from being a major virulence factor produced by *Streptococcus pyogenes*, the M protein is also the basis of a typing system developed by Rebecca Lancefield in 1928. M typing is a serological system based on antigen-antibody reaction, depending on the preparation of type-specific sera and extraction of the M protein from the surface of GAS [118]. From 1928 to 1966, 51 different M types were described and at the present, there are 83 GAS M serotypes serologically unique and encoded by unique *emm* gene sequences [71]. However, it was soon recognized that this typing technique presented two important disadvantages: the difficulty in the preparation and maintenance of the sera with a high

number of M types, each requiring a specific serum, and perhaps most importantly, the increasing number of isolates non-typeable by this method due to low expression levels of the M protein or lack of specific sera. To overcome these disadvantages, another typing method was developed, based on the production of antibodies against the opacity factor (SOF). Although not all *S. pyogenes* strains produce this apoproteinase, the opacity factor is antigenically specific and correlates with the M type. This was particularly important in the case of M sera for opacity-producing serotypes, that were of poor quality and difficult to prepare [136]. However, this did not solve the problem with M typing that was eventually superseded by *emm* typing.

The T protein, a surface protein of *S. pyogenes* with serological activity, is the basis of a different typing scheme, T-typing [92]. It is an agglutination test between the T antigen extracted from the cell wall upon treatment with pepsin or trypsin and a set of commercially available sera. Certain T types are associated with a specific M types [9, 10]. There are 25 different T types and unlike the M protein, the most conserved region of this protein is the amino-terminal region [108]. This typing method has a low discriminatory power and the number of non-typeable isolates is still considerable.

***emm* typing**

In the late 1980s, the resurgence of rheumatic fever and the increase in cases of invasive infections caused by GAS [53, 106, 182], as well as the number of isolates that could not be typed by the available phenotypic methods prompted a reassessment of the typing strategies leading to molecular approaches. The *emm* gene, that encodes the M protein, has a hypervariable region encoding the M serospecificity and it was soon demonstrated that the 5' sequences of the *emm* gene could efficiently predict the M type [11, 12, 111, 167], allowing the establishment of *emm* typing as an extension of the Lancefield M typing system. A Website was designed, allowing the comparison of the sequence resulting from the amplification by polymerase chain reaction and direct sequencing of the hypervariable region of the gene with all known *emm* sequences deposited (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm), using the BLAST algorithm and allowing the assignment of the *emm* type and subtype. There are currently more than 200 different *emm* types, of which some correspond to isolates that are M non-

typeable. The experience with M typing resulted in *emm* typing becoming the most widely used tool for epidemiological studies of GAS. However, it was recently shown that this typing technique is not sufficient to unambiguously identify GAS clones and should be complemented with other typing methods, such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST), for a better characterization of GAS clones [43]. The determination of *emm* restriction patterns, involving the multiple digestion of the PCR fragment used for *emm* typing with endonucleases can also be used (http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm)

Specific *emm*-types were already shown to correlate well with specific *emm* patterns [138] and it was also possible to establish an association between *emm* patterns and M protein class and the production of SOF; class I and SOF-negative belonged mostly to patterns A-D, while class II, SOF-positive to pattern E [19]. However, although the association between the *emm* patterns and the tissue of infection reflects a global trend, exceptions were reported [205].

Pulsed-field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis is widely used in epidemiological studies of *S. pyogenes*. It involves the enzymatic digestion of total DNA embedded in agarose, using an infrequently cutting endonuclease. The digested bacterial plugs are subjected to electrophoresis in which the polarity of the current is changed at previously specified time intervals, generating DNA digestion patterns. The comparison of these patterns allows the deduction of the genetic relationships between the isolates [156]. Figure 1.5 is an example of a typical *S. pyogenes* PFGE gel.

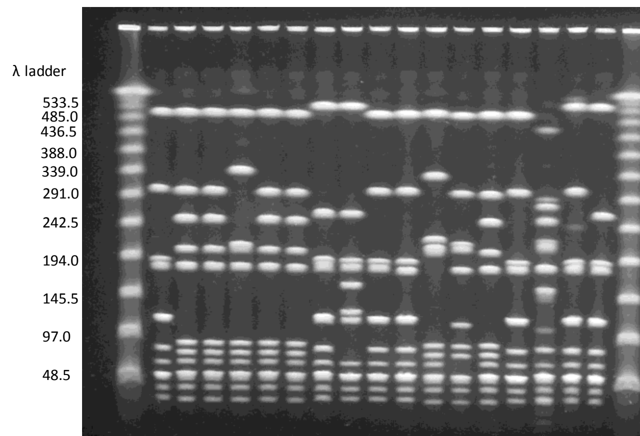

FIGURE 1.5: Pulsed-field gel electrophoresis (PFGE) profiles generated upon SmaI digestion of total DNA from *S. pyogenes*. Fragment sizes (kb) of the lambda (λ) PFGE marker (New England Biolabs, Beverly, USA) are presented.

The endonuclease used to generate the profiles is frequently SmaI, but isolates expressing the M phenotype with DNA that is resistant to digestion with SmaI were described [51]. To overcome this, it is common to use alternative enzymes, often SfiI, to characterize the two macrolide resistance phenotypes, but a direct comparison of the M and MLS_B clones is not possible when using different enzymes. The M isolates are resistant to the digestion with SmaI due to a methyltransferase encoded in the same genetic element that carries the *mef*(A) gene [79].

Guidelines for the interpretation of the DNA digestion patterns generated by PFGE were proposed by Tenover and colleagues in 1995. According to these criteria, isolates with the same pattern are genetically indistinguishable and represent the same strain; isolates with two to three band differences, resulting from a single genetic event (a point mutation or an insertion or deletion of DNA) are closely related. Differences of four to six bands (likely due to two independent genetic events) means that the isolates are possibly related and isolates are considered unrelated if the PFGE patterns differ in seven or more bands (corresponding to three or more genetic events)[204].

At the present time, data analysis is often accomplished by using commercially available software, such as Bionumerics (Applied-Maths, Sint-Martens-Latem, Belgium). The use of this software allows the normalization of the PGFE gels and the storage in a database, making it possible to compare the restriction patterns of large sets of isolates over time. The result is a dendrogram that represents the relationships between all the

isolates according to the criteria used for interpretation and comparison. Frequently, isolates sharing > 80% similarity on the dendrogram will be considered closely related, as supported by work with other streptococci [184]. PFGE is not only widely used in epidemiological studies of GAS, but it also presents a high discriminatory power [43].

GAS virulence has been related to the presence of bacteriophages and to horizontal gene transfer of large DNA segments thereby creating diversity in PFGE types, since alterations in the number and positions of the bands in the agarose gel can arise from these insertions on the bacterial chromosome [43, 185, 198]. Moreover, it is a very useful tool for short-term epidemiological studies, because the genetic variation indexed by this method accumulates relatively rapidly. This rapid accumulation of variation can represent a disadvantage for longer term epidemiological studies that would be better served by genetic variation that accumulates relatively slowly.

Multilocus sequence typing (MLST)

One of the major limitations of PFGE is the difficulty in comparing results obtained by different laboratories, which is particularly important in global epidemiology studies. To overcome this, a typing scheme was developed - multilocus sequence typing (MLST) - that takes advantage of the unambiguous nature and portability of DNA sequence data for the characterization of bacterial isolates. Internal fragments of seven housekeeping genes (their products are involved in vital functions, being present in every organism) are sequenced and an allele number is assigned by comparing to other sequences deposited in a database (www.mlst.net) [129]. An MLST scheme for *S. pyogenes* was developed [68] and 626 different STs were identified so far (spyogenes.mlst.net).

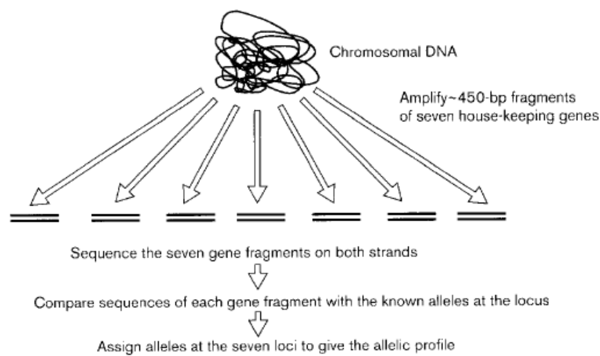

FIGURE 1.6: The multilocus sequence typing scheme: Amplification and sequencing of the internal fragments of housekeeping genes and comparison with known alleles, followed by assignment of the allele numbers and allelic profiles. Adapted from Spratt *et al*. [192]

Although it presents some limitations, such as the difficulty of the technique and the cost, it has many advantages, including its high resolving power, allowing in many instances the discrimination of isolates of the same *emm* type, and as variations within the nucleotide sequences of housekeeping genes accumulates relatively slowly, temporally separated isolates of the same ST can be recovered, which can be useful in long term epidemiological studies. The other advantage is the possibility of different laboratories comparing unambiguous results without the need to exchange strains. The clustering of isolates achieved by MLST is in good agreement with that obtained using other techniques [43, 68].

Based on MLST results, the clonal relationships between the isolates can be inferred using the eBURST algorithm that divides MLST data into groups of related isolates and clonal complexes, providing information about the founding genotypes or the likely patterns of evolutionary descent within the clusters [76]. This algorithm is based on a simple model: a clonal complex arises from the increase in frequency of the founding genotype (due to a fitness advantage or random genetic drift) and from its diversification. In terms of MLST, the descendants of the founder will present the same allelic profile but over time diversification will also occur and variants in one of the seven alleles (single locus variants - SLVs) will arise. Eventually these SLVs will diversify further, resulting in double locus variants (DLVs) or triple-locus variants (TLVs) and so on. Using the eBURST rules, it is possible to identify potential links between STs that correspond to the most probable pattern of descent, within each clonal complex [76]. However, the eBURST algorithm is not globally optimized and such links within the clonal complexes can violate the rules proposed. To overcome this, a globally optimized implementation of eBURST

was developed - goeBURST [82]. A software implementation of this algorithm is currently available (http://www.phyloviz.net), with the advantage of integrating additional information such as clinical or patient demographic data or other isolate information (such as other phenotypic and molecular characteristics) [83].

Superantigen gene profile

The profiling of the superantigen (SAg) gene content of GAS isolates - toxin profiling - has been used as a molecular typing method, since it allows further discrimination of GAS isolates of the same *emm* type. As mentioned above, some of the superantigens are encoded on temperate bacteriophages (SpeA, SpeC, SpeH, SpeI, SpeK, SpeL, SpeM) and the exchange and integration of these prophages among GAS isolates must play an important role in the genomic diversification of the species and in the emergence of clones associated to toxin-mediated diseases, such as scarlet fever or invasive GAS disease [84, 137, 221].

A recent method of SAg profiling was developed, using two multiplex PCR reactions that allow the detection of 13 gene fragments (11 superantigens - SpeA, SpeC, SpeH, SpeI, SpeK, SpeL, SpeM, SpeG, SpeJ and SMEZ - and SpeB and SpeF, used as positive controls) covering all the known allelic variants of the genes [84]. A similar typing method that also claims to include all the superantigens, but also other genes encoding virulence factors was developed, but is was already suggested that the profiling of additional virulence factors did not bring additional information [34]. Using SAg profiling, a strong association between the complete profile and *emm* type was reported and it was also suggested that variation of the SAg profile in the population occurs at a faster rate than some of the usually used typing methods like *emm* typing or MLST, being suitable in the identification of clones associated with specific properties, like the association to a specific disease or enhanced virulence [63, 85, 86, 222].

Whole-genome sequencing

The importance of recombination as a key factor in bacterial evolution has been recognized, and it has also been suggested that recombination rates in bacteria may be higher than mutation rates [74, 125, 191]. Furthermore, when recombination involves DNA portions that are larger than the internal gene fragments analyzed by MLST, the result will be the identification of the same alleles in strains with different genetic backgrounds [42]. To overcome this and to complement and improve the typing techniques described above, whole genome sequencing methods have been applied to the study of *S. pyogenes*, offering highly sensitive and unequivocal comparison at the single nucleotide level [14].

Up to now (December, 2013), the full genomes of 19 strains are available. (http://www.ncbi.nlm.nih.gov/genome/genomes/175). Each genome is approximately 1.9 Mb in size, and about 10% of the overall gene content is encoded on variably present exogenous elements, such as prophages and integrative conjugative elements [15]. With the availability of these genomes, many virulence factors were identified, novel host-pathogen interactions were elucidated and several virulence factor regulatory pathways were studied, providing new insights into the molecular pathogenesis of *Streptococcus pyogenes* [148].

The recent advances in next generation sequencing technologies, offering the opportunity to obtain complete or nearly complete genome sequences of large numbers of individual strains will allow the establishment of genetic differences, undetectable by other typing techniques, with recognized advantages for both outbreaks and long-term epidemiological studies [14, 16, 42].

Although less frequently, the epidemiological characterization of GAS was done using other methods, such as Multilocus Enzyme Electrophoresis (MLEE), vir typing, ribotyping, Random Amplified Polymorphic DNA (RAPD) and Fluorescent Amplified-Fragment Length Polymorphism Analysis (FAFLP) [75, 99].

1.7 Antimicrobial resistance and treatment of GAS infections

The treatment of patients with acute pharyngitis should be done with an antibiotic at an appropriate dose for a period expected to eradicate the microorganism from the pharynx [188]. Among the antibiotics available for the treatment of GAS pharyngitis are beta-lactams (such as penicillin, ampicillin and amoxicillin), many cephalosporins, macrolides and clindamycin [31]. Due to its efficacy, safety, narrow spectrum and cost, penicillin remains the treatment of choice for this type of infections [188]. The duration of the treatment with oral penicillin is usually 10 days, mainly to protect against the complication of acute rheumatic fever [4]. However, other antibiotics taken for a shorter duration such as cephalosporins or macrolides may have a comparable effect to penicillin taken for 10 days, overcoming the possible low compliance of long antibiotic course [4]. In Portugal, where oral penicillin is not available, macrolides and lincosamides, which are often recommended as suitable alternatives for patients allergic to penicillin [31], are an option with the additional advantage of having an oral route of administration.

1.7.1 β-lactams

The mechanism of action of β-lactam antibiotics involves the binding and inactivation of bacterial proteins that are responsible for cell wall synthesis, the penicillin binding proteins (PBPs) [44].

In *S. pneumoniae*, penicillin resistance in natural populations is associated to mosaic genes, resulting from the replacement of segments of PBPs of susceptible strains by homologous blocks from resistant strains, most likely by natural transformation. In spite of extensive use of penicillin in the treatment of GAS infections, there are no reports of β-lactam resistance detected *in vivo* in *S. pyogenes* isolates and one of the reasons for this could be the fact that PBPs of *S. pyogenes* contain no lengthy regions of similarity with genes from other streptococci, making it unlikely that the acquisition of penicillin resistance arises by homologous recombination with genes from other species [78]. Based on the creation of penicillin-resistant laboratory mutants, it was already suggested that in the case of *S. pyogenes*, the emergence of penicillin resistance could lead to marked changes in the biology of the microorganism including the expression of low-affinity

PBPs, physiological defects, poor growth rates, decreased production of M protein and morphological abnormalities [94, 105].

1.7.2. Tetracyclines

Tetracyclines bind reversibly to the 30S ribosomal subunit, blocking the binding of the tRNA, inhibiting protein synthesis. Resistance to tetracycline arises upon genetic acquisition of *tet* genes. Four variants of this gene were already reported in *S. pyogenes*, *tet*(M) and *tet*(O), that encode ribosomal protection proteins and less frequently, *tet*(K) and *tet*(L), encoding efflux pumps [46].

Although tetracyclines are not usually used in the treatment of GAS infections, their use as animal growth factors can promote the emergence of resistance in human pathogens [46]. Tetracycline- and macrolide-resistance genes are often carried in the same genetic elements and the most frequent associations are *tet*(M) and *erm*(B) or *mef*(E) genes [33, 35, 82], as well as *tet*(O) and *mef*(A) or *erm*(A) genes [38, 62, 91]. There are several genetic elements carrying these genes, and some are found among other species of the genus *Streptococcus* [66, 210], with the ability to spread within streptococcal populations [100, 123]. Given this, tetracycline resistance surveillance studies are important and it was already suggested that tetracycline use could act as a selective force driving macrolide resistance in *Streptococcus pyogenes* [151].

1.7.3 Macrolide and Lincosamides

Macrolides and lincosamides are chemically unrelated but share many biological properties, such as the mechanism of action and mechanism of resistance, antimicrobial activity and pharmacological properties. Erythromycin, produced by *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*), is a 14-member macrocyclic lactone ring, attached to two sugar moieties. Newer macrolides, such as azithromycin or clarithromycin, are derived from erythromycin and have a greater spectrum of action, better oral absorption, longer half-life and fewer side effects. Lincosamides are devoid of the lactone ring. Lincomycin was the first lincosamide isolated from a microorganism and its chemical modification produced clindamycin, with higher absorption upon oral administration [194].

Macrolides and lincosamides inhibit protein synthesis due to the binding of the antibiotic to the 50S ribosomal subunit that results in stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation and chain termination [172].

1.7.4 Streptogramins

Streptogramins are natural antibiotics, consisting of an association between two types of molecules, group A and group B streptogramins [175]. Alone, each compound exhibits a moderate bacteriostatic activity, but in combination they can produce a bactericidal effect. Streptogramin B acts on the 50S ribosomal subunit, competing with macrolides for the same binding sites; resistance to both classes of antibiotics arises through the same mechanisms. Type A streptogramins blocks peptide bond formation, inhibiting the peptidyl transferase reaction directly [98].

1.7.5 Macrolide resistance mechanisms in GAS

There are two described mechanisms used by *S. pyogenes* to resist the bacteriostatic action of macrolides: target site modification, by methylation or mutation that prevents the binding of the antibiotic to the ribosome and active efflux of the antibiotic. These mechanisms have already been described in macrolide and lincosamide producers, to protect themselves from the action of the antibiotics [124].

Methylation was the first mechanism of macrolide resistance and it is due to posttranscriptional modification of the 23S rRNA by a methyltranferase [213]. In general, genes encoding these methylases are designated by *erm* (erythromycin ribosome methylase) and several classes of *erm* genes have been described [172]. As the binding site in the 50S ribosomal subunit for erythromycin overlaps the binding site of the newer macrolides, as well as lincosamides and streptogramin B antibiotics, the modification by methylation reduces the binding of most macrolides, lincosamides and streptogramin B, resulting in the MLS_B phenotype [215]. Expression of MLS_B resistance can be constitutive or inducible, generating the $cMLS_B$ or the $iMLS_B$ phenotypes, respectively. In the case of the $iMLS_B$ phenotype, bacteria produce inactive mRNA that becomes active only in the presence of a macrolide inducer. In the presence of the inducer (usually erythromycin),

there are mRNA rearrangements, allowing the ribosomes to translate the methylase coding sequence [124]. On the contrary, in bacteria expressing the $cMLS_B$ phenotype active methylase mRNA is produced in the absence of an inducer. Phenotipically, $iMLS_B$ isolates present reduced clindamycin inhibition zone proximal to the erythromycin disk in the double disk test, whereas a regular circular shape is present around the clindamycin disk when the disks are placed apart [214].

Many of the *erm* genes are associated with conjugative or non-conjugative transposons which are often associated with other antibiotic resistance genes, although some have been found in plasmids [37, 172]. These transposons, with the capacity to transfer the resistance traits to susceptible isolates by conjugation [89, 90], have a wide host range, which can explain the observation that clinical isolates from many different bacterial species carry these *erm* genes [172].

The other mechanism, responsible for resistance to 14- and 15-membered macrolides and susceptibility to 16-membered ring macrolides, lincosamides and streptogramin B (the M phenotype), is mediated by a membrane associated protein that pumps the antibiotic out of the cell, keeping intracellular concentrations low and preventing the binding of antibiotics to the ribosome. This membrane associated pump is encoded by *mef* genes (macrolide efflux) [200]. Several *mef* genes have already been characterized of which *mef*(A) is the most frequently found [49]. The *mef*(E) determinant, first described in *S. pneumoniae*, shares 90% DNA and 91% aminoacid identity with *mef*(A) [202] and its presence in *S. pyogenes* isolates was already confirmed [32]. The discrimination between *mef*(A) and *mef*(E) is epidemiologically important because they are carried in different genetic elements and their dissemination is also different [61, 62]. Other *mef* genes were found in *S. pyogenes*, such as *mef*(I), *mef*(O), a *mef* mosaic variant, in which the 5’ region was identical to that of *mef*(A) and the 3’ region was identical to that of *mef*(E), and another *mef* variant [32, 178]. The *mef*(A) gene is usually associated with a chimeric element composed of a transposon inserted into a prophage [7], while the *mef*(E) genes are often associated with mega elements, in association with other resistance genes in composite transposons [62]. The genetic elements carrying the *mef* genes have already shown the ability to be transferred between different *S. pyogenes* isolates [90, 110].

Other mechanisms of resistance such as alterations of ribosomal proteins were also described, but are not frequently observed in GAS isolates responsible for infections and have little clinical impact [25].

1.7.6 Epidemiology of macrolide resistance

Less than 20 years after the isolation of erythromycin from *Saccharopolyspora erythraea* in 1952 and its introduction in the clinical practice, resistance to this antibiotic became apparent in some clinical isolates. In 1968, the first group A streptococci resistant to erythromycin were reported in England, in the Unites States and in Canada [65, 112, 177]. Since then, increases in macrolide resistance were reported in many countries, although each country may retain specificity regarding resistance rates, prevalence of the macrolide resistance phenotypes and phenotypic and molecular characteristics of the resistant isolates.

A relationship between the decrease in macrolide resistance and the decrease in macrolide consumption was already demonstrated [3, 17, 59, 211]. Some studies showed an association between specific macrolides (intermediate acting and long-acting macrolides) in the selection of resistance and of a particular macrolide resistance phenotype [131, 144]. However, the relationship between macrolide consumption and resistance is not always so obvious. In Slovenia, after an increase in macrolide consumption (mainly long-acting macrolides), paralleled by a significant increase in the macrolide resistance rate from 0 % to 7.4% in the period 1994-1999, resistance rates doubled in *S. pyogenes*, reaching a value of 20% in 1999-2004, despite a 21% decrease in macrolide consumption [47, 48]. In Spain, a decrease in macrolide resistance rate was reported with no significant changes in macrolide consumption [157]. Macrolide use must be the main driver of macrolide resistance in *Streptococcus pyogenes*; however, natural clonal fluctuations may also play a role.

In Spain, high macrolide resistance rates have been reported. Between 1996 and 2007 in four studies, including 2,287 *S. pyogenes* isolates, the antimicrobial resistance rates and phenotypes of resistance, as well as their temporal trends were analyzed. Over this 11-year period, 19% of the isolates were resistant to erythromycin, with a clear predominance

of the M phenotype (64.5%). Among the MLS_B isolates, the great majority expressed the $cMLS_B$ phenotype (94.9%), while the $iMLS_B$ phenotype was expressed by only 5.1% of the isolates. In this period, a temporal increasing trend in the prevalence of the MLS_B isolates was reported (from 14% in 2001-2002 to 35.5% in 2006-2007), probably due to the replacement of macrolide resistant clones. Interestingly, a decreasing macrolide resistant trend was detected in this same period (24.3% in 2001-2002 to 19.0% in 2006-2007, $p<0.001$), that was undetectable when analyzing the whole study period [161]. A higher resistance rate was reported in this country, between 1994 and 2006 (33%), still with a clear dominance of the M phenotype (77%). Isolates presenting the MLS_B isolate presented mostly the constitutive form of this phenotype (20%) while 3% expressed the $iMLS_B$ phenotype. Among the $cMLS_B$ isolates, *erm*(B) was the most prevalent resistant determinant and *erm*(TR) was associated to the $iMLS_B$ phenotype. The M isolates carried *mef*(A/E), with no distinction made between these two genetic elements [176]. However, this study included GAS isolates recovered from a wide spectrum of clinical manifestations, including severe GAS infections, skin infections, scarlet fever, rheumatic fever and tonsillo-pharyngitis, as well as asymptomatic carriers, and macrolide resistance rates are different in different types of infection [55, 86].

Another survey in Spain including mostly pharyngeal isolates detected a high macrolide resistance rate (21%), with annual variations of 14.3% to 28.9% between 1999 and 2005 and an increase in the prevalence of MLS_B isolates was also noted, from 3% in 1999 to 13% in 2005. Although there were fluctuations in the prevalence of isolates presenting the M phenotype, their tendency since 2003 was to decrease [162].

Temporal and geographical variations in macrolide resistance rates and phenotypes are frequent and sometimes local variations within the same country can occur. In Cantabria, Spain, macrolide resistance reached a maximum of 53.6% between July and December of 2002, but a rapid decrease in the last quarter of 2003 was reported and in 2004 the macrolide resistance rate was as low as 3.7%. In this period there was also a rapid inversion of macrolide resistance phenotypes, with a decrease in the prevalence of the M phenotype, paralleled by an increase in the proportion of isolates expressing the MLS_B phenotype, without significant changes in macrolide consumption [157].

In Greece, macrolide resistance in GAS has also reached high values. In a survey conducted in Western Greece, between January 1999 and December 2002, including only GAS recovered from children with tonsillo-pharyngitis, a resistance rate of 24% was reported, without a clear dominance of any of the macrolide resistance phenotypes. However, it was not known if the macrolide resistance rates or the prevalence of the macrolide resistance phenotypes were stable throughout the study period [93]. Another study, carried out between January 2003 and December 2006, detected a lower value of resistance (15%), as well as an increase in macrolide resistance rate from 12% in 2003 to 19% in 2006. Once again, there was no considerable dominance of any of the macrolide resistance phenotypes (53.7% expressed the M phenotype and 46.3% the MLS_B phenotype). However, this study included mostly tonsillo-pharyngitis patients, but also other types of infection from only one Greek hospital and when considering pharyngeal isolates only, macrolide resistance was significantly higher [140].

A more recent survey revealed a macrolide resistance rate of 24%, stable during the study period (January 2007 and June 2009), but an increase in the prevalence of the MLS_B phenotype was noted with a concomitant decrease in the prevalence of the M phenotype. In this work, the authors attributed the appearance of resistant isolates to the emergence of particular clones [133].

In Italy, macrolide resistance in *S. pyogenes* between 1993 and 1996 remained low, below 5%. However, from 1995 to 1996 an increase in macrolide resistance from 2.6% to 17.1% was reported, and the M phenotype was the most frequent, with 73% of the isolates expressing this phenotype [23]. In 2000, high macrolide resistance rates (35.8%) were reported, although this study included only 127 GAS recovered from the same hospital. The most frequent macrolide resistance phenotype was MLS_B, expressed by 83 isolates (65%) [64]. More recent data reported a significant decrease in macrolide resistance from 28.1% in 2001 to 15.6% in 2006. The overall macrolide resistance in this period was 22.6% (n=320), without the dominance of any of the macrolide resistance phenotypes. This study was conducted in a central Italian region and the authors found a correlation between this decrease and the consumption of a specific class of macrolides, namely long-acting macrolides [144].

Lower values of macrolide resistance were reported in Germany. Between 1999 and 2002 and between 2002 and 2004, the macrolide resistant rate was constant, with a value of 14%, but from one period to another, an increase in the prevalence of isolates expressing the M phenotype was reported [165, 166]. In the first study, 55.6% of the isolates presented the M phenotype and carried the *mef*(A) gene; iMLS$_B$ isolates represented 31.5% of the population (carrying the *erm*(A) gene) and only 13% of the isolates presented the cMLS$_B$ phenotype and carried the *erm*(B) gene [165]. In 2002-2003, a dramatic increase in the prevalence of isolates presenting the *mef*(A) genotype was reported, reaching the value of 94%, in spite of the same resistant rate [166]. However both studies included a low number of isolates (possibly due to short study periods) and one included throat and skin isolates [165], while the other included solely isolates from community-acquired respiratory tract infections [166]. In recent years, a decrease in the macrolide resistance rate was reported to 8.2% (n=29) in 2005-2006 [33] and to 2.6% (n=9) in 2006-2009 [73]. However the latter was performed in one medical centre and can represent a particular situation of lower macrolide resistance that the authors attributed to a more restrictive use of macrolides in that region [73]. The number of resistant isolates is low in both studies, without any dominant macrolide resistant phenotype.

In France, macrolide resistance increased from 6% (n=93) to 22% (n=72) between 1996-1999 and 2002-2003. Both studies included pharyngeal isolates recovered from pediatric patients. The MLS$_B$ phenotype was the most prevalent, although in 2002-2003, the difference in the prevalence of both phenotypes was more evident. The M isolates carried the *mef*(A) gene and the great majority of the MLS$_B$ isolates carried the *erm*(B) resistant determinant [24, 25]. However, from October 2009 to May 2011, only 19 isolates from a collection of 585 GAS isolates collected from children with pharyngitis were resistant to erythromycin, indicating a decrease in macrolide resistance in France, reaching the value of 3.2%, that was attributed by the authors to a decrease in antimicrobial consumption in this country [59]. A significant decrease in macrolide resistance, significantly correlating with a decrease in antimicrobial consumption was also reported in Belgium, where the prevalence of macrolide resistant *S. pyogenes* decreased from 13.5% to 3.3% during 1999-2006 and remained low from 2006 onward. Interestingly, this decrease was accompanied by an increase in the proportion of *erm*(A) positive isolates among the

resistant population, from 1.2% (n=81) to 76.6% (n=36) in 2009 and a decrease in the proportion of *mef*(A) and *erm*(B) isolates [211].

In Finland, there was also a statistically significant decrease in macrolide resistance rate among GAS isolates, after a reduction in the use of macrolide antibiotics. An increase in macrolide resistance was reported until 1993, when resistance rates reached high values (19%), but resistance diminished to a value of 8.6% in 1996 [183]. In a study conducted between 1994 and 1995, the majority of the isolates expressed the M phenotype (61%, n=242), and only 4 of the MLS_B isolates presented the constitutive form of this phenotype, while 150 isolates expressed the $iMLS_B$ phenotype [110]. In the period between 1997 and 2001, macrolide resistance in GAS presented a decreasing trend, although it did not reach statistical significance [17]. In 2004-2005, macrolide resistance in Finland was low, below 5%, although no information about the prevalence of macrolide resistance phenotypes was available [168]. Low macrolide resistance rates were also reported in Norway, the Netherlands and Sweden [127, 168].

Besides the geographical and temporal differences in macrolide resistance rates and in the prevalence of particular macrolide resistance phenotypes and genotypes, differences in the dominant *emm* types were also documented. Usually, the majority of these *emm* types shared the same resistance determinants and presented the same ST, which suggests a wide geographical dissemination of a few clones. However, isolates fully susceptible to macrolides that share the same pulsed-field gel electrophoretic profiles (PFGE) or the same sequence types (STs) as these major clones have been described, and several resistant determinants have been associated with each resistant lineage [59, 64, 130, 133, 162, 165, 201, 223]. Taken together, these data imply that independent acquisition of resistance genes by the same prevalent clones followed by local expansion could have also played a role in conditioning the successful macrolide resistance phenotypes and clones in particular geographic locations.

An association between certain *emm* types and macrolide resistance was already documented [2, 46, 110, 119, 136, 182], but in most of these studies the characterization of the *S. pyogenes* isolates was limited to *emm* typing. Moreover, it is still not clear if the macrolide resistant population has its own dynamics or if this is simply the reflex of

molecular characteristics of the general GAS population. The question of whether some genetic backgrounds are more prone to acquire and carry macrolide resistant genes or the high prevalence of a particular *emm* type in the macrolide resistant population is just the result of an equally high prevalence in the general population remains unanswered.

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Aims of the Thesis

Macrolides are suitable alternatives for the treatment of infections caused by *Streptococcus pyogenes*. There are large geographical and temporal differences in macrolide resistance rates in *S. pyogenes* causing tonsillo-pharyngitis. Several studies have found a relationship between antibiotic consumption and macrolide resistance, with some specific classes of macrolides being more implicated than others in the selection of resistance and of particular resistance phenotypes. However, natural fluctuations of the clones can also play a role in the prevalence of macrolide resistance rates, although the contribution of each factor remains unclear.

The main purpose of the work presented in this thesis was the characterization of *S. pyogenes* associated with tonsillo-pharyngitis in Portugal, with a special focus on macrolide resistance. This included the determination of macrolide resistance among GAS causing tonsillo-pharyngitis, as well as their macrolide resistance phenotypes and evolution in a large period of time and the characterization of the clonal composition of the population. The knowledge of the macrolide resistance rates is important for the empiric treatment of GAS infections, since they provide an alternative to penicillin. Understanding the dynamics of the resistant population is equally important, in order to track the dissemination of specific clones in close geographic areas or to identify particularly successful clones. The knowledge of the molecular characteristics of the population will also provide some insights into the contribution of fluctuations of the clonal composition to the overall macrolide resistance rate. For this purpose, the macrolide susceptibility among GAS isolates recovered from patients with tonsillo-pharyngitis in several laboratories located throughout Portugal was determined and a collection of 620 macrolide resistant GAS were studied by phenotypic and molecular techniques, which included the determination of macrolide resistance phenotypes and genotypes, T typing, *emm* typing, PFGE restriction profiling and MLST. All the genetic lineages identified were compared with the ones described in other European countries.

Another important contribution for the main purpose of this thesis involved a study to determine the relative contributions of geographic dissemination of macrolide resistant clones and genetic acquisition of macrolide resistance genes followed by local spread. To do this, a collection of 803 GAS isolates, isolated between 2000 and 2005, representing

50% of the total pharyngeal isolates recovered in Portugal was characterized and compared. All the isolates were characterized using T typing, *emm* typing, PFGE profiling and MLST. Statistical tests were used to ascertain the differences between the two populations.

Another important goal of this thesis was to characterize the *S. pyogenes* isolates causing scarlet fever in Portugal, given the recent outbreak reports. The large collection of pharyngeal isolates available allowed us to use the same approach used to study macrolide resistant GAS - a comparison between scarlet fever isolates and pharyngitis isolates, in an attempt to identify molecular markers of this syndrome. Besides the usual typing techniques indicated above, SAg profiling was also performed, given the frequent, although contradictory association of some exotoxins produced by *S. pyogenes* to scarlet fever.

Lastly, we aimed to find out if the strategy used in the microbiology laboratory of isolating and subculturing one single colony from the primary swab plate was the most acceptable for antimicrobial susceptibility testing and other epidemiological studies. This study was prompted by recent reports of heterogeneity among colonies recovered from a single patient. For this purpose, we analyzed colonies isolated from the same primary plate of 35 pharyngitis patients, from the same hospital - originating a total of 321 isolates, by antimicrobial susceptibility testing and *emm* typing.

CHAPTER 2

INTRAHOST VARIATION IN INFECTION

INTRODUCTION

The human oropharynx is heavily colonized and it is the site for carriage of many important human pathogens, including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Moraxella catarrhalis* and *Staphylococcus aureus* [15].

In the microbiology laboratory, the identification of the bacterial causative agent of tonsillo-pharyngitis involves the culture of the throat swab in blood agar plates [3]. The presence of beta-hemolytic colonies among other normal flora in a throat culture, associated to a diagnosis of tonsillo-pharyngitis is indicative of *Streptococcus pyogenes* as the ethiological agent involved. In order to identity the agent and further characterize the bacteria, it is standard procedure to isolate and subculture one single colony, with the underlying assumption that the infection is caused by a single clone. However, it is well known that different genotypes or phenotypes can be found within a bacterial population in carriage and infection [20, 24].

Co-colonization, or the presence of more than one strain in the nasopharynx seems to be required for horizontal gene transfer between different pneumococcal strains [4, 16, 21], which may lead to changes in capsular serotype or antimicrobial susceptibility. Using culture-based techniques, *S. pneumoniae* co-colonization rates were determined to range from 1% to 20% [9, 12, 21, 25], and using molecular techniques higher rates were reported [25]. Most of these studies described the serotype diversity among *Streptococcus pneumoniae* [4, 9, 12, 25], while others also addressed the genetic diversity of these isolates and found differences in the serotype, PFGE type, prophage content and antimicrobial susceptibility patterns in carriage [21]. It was recently demonstrated that genetic exchange in conditions similar to those found *in vivo* is extremely efficient [16]. Moreover, it is well known that horizontal gene transfer events are important in the emergence of novel virulence traits as well as in the dissemination of antimicrobial resistance determinants and epidemiological studies indicate that the source of these antimicrobial resistance genes seems to be the other colonizing strains, rather than invasive isolates [7]. However, contrary to *S. pyogenes*, *S. pneumoniae* is naturally transformable, incorporating foreign DNA by transformation, in which the horizontal gene transfer between different strains co-existing in the same niche is more likely to occur [16].

In *Streptococcus pyogenes*, there are few studies addressing the occurrence of more than one strain in a single host. In a study conducted in an area where acute tonsillo-pharyngitis is endemic, recovering 5 isolates from each sample, a single clone of *S. pyogenes* was detected in each case of infection using pulsed-field gel electrophoresis [19]. Also in the oropharynx of healthy carriers, a single clone of *S. pyogenes* was detected giving the first indication that co-colonization is rare [19]. However, antibiotic resistance heterogeneity in streptococcal isolates was recently described in different *S. pyogenes* colonies from the same throat swab [26]. This study included 16 pediatric patients, and heterogeneity was found both in antimicrobial susceptibility pattern (in 3 patients) and *emm* subtype (in one patient). The differences in antimicrobial susceptibility included the acquisition of the transposon Tn*916*, carrying the *tet*(M) gene, conferring tetracycline resistance in one isolate among nine susceptible isolates and the gaining of the transposon Tn*1207*, originating macrolide resistance through the action of the *mef*(A) gene [26]. In spite of this, all the resistant and susceptible isolates from the same patient shared the same *emm* type and multilocus sequence type (MLST) sequence types (ST). In the only case where differences were observed solely on the *emm* subtype level, all the isolates shared the same ST and antimicrobial resistance profile. This heterogeneity among isolates of the same genetic background raised the question of whether culture-based techniques that rely on the assumption that an infection is caused by a single clone are adequate to perform antimicrobial susceptibility testing and epidemiological studies. According to the authors, if only a minority of colonies carries resistance genes, the classical antimicrobial susceptibility testing would consider the strain as susceptible, but the proliferation of the resistant isolates after initiation of antibiotic treatment could have as a consequence a therapeutic failure. Moreover, single-colony based epidemiological studies could be measuring antimicrobial resistance rates that were underestimated [26].

The aims of this study were to evaluate the potential bacterial molecular heterogeneity and diversity in antimicrobial resistance profiles in GAS isolates recovered from single throat swabs of patients associated with a diagnosis of tonsillo-pharyngitis, in a large teaching hospital in Lisbon.

MATERIALS AND METHODS

Bacterial isolates

Bacterial isolates were recovered from 44 patients with a diagnosis of tonsillo-pharyngitis, from September 2006 to April 2007 in the microbiology laboratory of Hospital de Santa Maria in Lisbon. For each throat swab culture, ten β-hemolytic colonies (presumably GAS colonies) were selected from the primary plates and subcultured to a secondary plate for conservation and further testing. In some cases, it was not possible to isolate 10 colonies for some primary plates; when less than 8 colonies were recovered, the primary plate was excluded from the study (n=9). The final collection included 321 GAS isolates, from 35 patients, in which 15 samples originated 10 isolates, 11 samples 9 isolates and 9 samples 8 isolates.

Antimicrobial susceptibility testing and macrolide resistance phenotype

Susceptibility to erythromycin, clindamycin, tetracycline, norfloxacin and trimetropim- sulfametoxazole (Oxoid, Basingstoke, UK) was tested using disk diffusion according to CLSI recommendations [6]. The macrolide resistance phenotype was determined according to a double disk test previously described [18].

DNA extraction

Total bacterial DNA was isolated according to the methodology described by the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm).

emm typing

emm typing was performed as described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Amplification products were purified using the High Pure PCR purification kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and sequenced using primer emmseq2, and the DNA sequences were searched against the *emm* sequences deposited in the CDC database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

RESULTS

Antimicrobial susceptibility testing

Among the colonies isolated from each of the primary plates, there were no differences in the antimicrobial resistance profiles. Given this homogeneity, results of antimicrobial susceptibility testing will be presented by sample and not by individual colonies.

Among the 35 samples, erythromycin resistance was found in isolates from 5 samples and clindamycin in isolates from 3 isolates samples. Three samples yielded the $cMLS_B$ phenotype, while 2 presented the M phenotype. Tetracycline resistance was expressed by isolates from one sample. Two samples presented isolates expressing resistance to norfloxacin and all the isolates were susceptible to trimethoprim-sulphamethoxazole.

emm typing

Similarly to the antimicrobial resistance profiles, there were no differences in the *emm* type presented by all the colonies from the same primary plate. Furthermore, the amplification and sequencing of the *emm* gene revealed the same *emm* subtype among GAS isolates originated from the same plate.

The most frequent *emm* type was *emm*4.0 (20%, n=7), followed by *emm*44.0 (14%, n=5). The characteristics of the samples included in this study, are summarized in Table 2.1

TABLE 2.1: Characteristics of the 35 samples included in this study

emm type (no. of samples)	Samples (no. of colonies)	Resistance profile (no. of samples)[a] [Macrolide resistance phenotype (no. of samples)]
4.0 (7)	2(10); 2(9); 3(8)	Fully susceptible (7)
44.0 (5)	3(10); 2(8)	Fully susceptible (5)
1.0 (4)	2(10); 1(9); 1(8)	Fully susceptible (4)
12.0 (4)	3(10); 1(8)	Fully susceptible (3); Ery^R Cli^S Te^S Nor^S (1) [M(1)]
28.0 (3)	1(10); 1(9); 1(8)	Fully susceptible (1); Ery^R Cli^R Te^S Nor^S (2) [MLS_B (2)]
75.0 (3)	1(10); 2(9)	Fully susceptible (2); Ery^R Cli^S Te^S Nor^S (1) [M(1)]
89.0 (3)	2(10); 1(9)	Fully susceptible (3)
6.0 (2)	1(10); 1(9)	Fully susceptible (1); Ery^S Cli^S Te^S Nor^R (1)
6.4 (1)	1(9)	Ery^S Cli^S Te^S Nor^R (1)
11.0 (1)	1(9)	Ery^R Cli^R Te^R Nor^S (1) [MLS_B (1)]
3.1 (1)	1(9)	Fully susceptible (1)
87.0 (1)	1(8)	Fully susceptible (1)

[a)] All the samples were susceptible to trimethoprim-sulphamethoxazole, so it was not discriminated. Ery - erythromycin, Cli - clindamycin, Te- tetracycline, Nor - norfloxacin, R - resistant, S- susceptible

DISCUSSION

Differences in the antimicrobial resistance profiles among colonies recovered from the same patient, with a diagnosis of tonsillo-pharyngitis were recently reported, implying that the single colony strategy used in the microbiology laboratory could lead to an incorrect measurement of antibiotic resistance rates, as well as failures in the treatment. All the differences detected were found among isolates presenting the same genetic background, implying that the heterogeneity arose from horizontal gene transfer of some resistant determinants to a minor population of the infecting organism [26].

Our results are contrary to these, even with a larger collection. We detected no differences in antimicrobial resistance profile among all the colonies isolated from each primary plate, which suggested that in our collection, horizontal gene transfer is not frequent in the course of an infection. Our results are in agreement with another study which demonstrated that GAS has a clonal nature in carriage and infection. In that study all the colonies from the same throat swab were analyzed by PFGE. Although we have previously shown that PFGE is a good typing technique with high resolving power, differentiating macrolide resistant and susceptible isolates [22], it is possible that some differences remain undetected using this typing method, such as *emm* type or subtype.

All the isolates in the Belgium study, in which differences in the antimicrobial resistance profiles or other molecular differences, such as a different *emm* subtype were found, presented *emm*3 [26] and in our collection only one isolate expressed this *emm* type. Furthermore, we have previously showed that *emm*3 is highly prevalent in the general GAS population in Portugal, but very rare among the resistant population [22]. In other studies, this *emm* type was reported to be rarely associated with macrolide resistance [1, 28]. The infrequent association of macrolide resistance to *emm*3 isolates could be related to an intrinsic feature of these isolates, rendering them more resistant to acquisition of macrolide resistance determinants, although this remains to be established. Macrolide and tetracycline resistance determinants are carried in mobile genetic elements and some studies indicate that M proteins could function as barriers to horizontal gene exchange [5, 23]. However, recent studies suggested that extensive lateral gene transfer occurred among GAS isolates of different M types [2, 27]; the role of M protein as a barrier to horizontal gene exchange is still to be completely elucidated.

In the literature there are some reports of intrahost sequence variation of some GAS proteins. The M protein was soon recognized to exhibit large differences in size and antigenic variation between different serotypes [8, 14]. This size variation, believed to be generated by homologous recombination between intragenic repeats, was detected among laboratory cultures of group A streptococcal strains [13] and it was also reported in a single patient, diagnosed with rheumatic fever caused by GAS [11], although the multiple colonies analyzed were obtained in consecutive days.

More recently, a study including GAS recovered from pharyngitis patients detected intrahost sequence variation of the gene that encodes for another protein - the streptococcal inhibitor of complement (Sic), present mostly in *emm*1 isolates [17]. This study detected mixed infections with strains containing 2 distinct *sic* alleles in five out of the 20 patients analyzed; for each patient, a total of 100 colonies were screened. In all cases where mixed infections were detected, there was a major (>90%) and a minor (<10%) *sic* allele. Within the same host, all the isolates had the same genetic background, suggesting that the variation in the *sic* gene arose from one single infecting *emm*1 organism [17].

According to the authors, these differences in size and sequence variation of the proteins could provide a survival advantage to the organism. In the case of the M protein, differences in size would lead to antigenic differences, due to different number of epitopes available per M molecule. Likewise, the Sic protein was already shown to contain epitopes detected by human antibodies [10], and its variation resulting in mixed infections could potentially contribute to pathogen survival by immune avoidance [17]. Although we have found no heterogeneity in our collection regarding antimicrobial resistance profiles or *emm* type and subtype, it would be interesting to analyze the size and sequence of the M proteins, in order to detect any intrahost size variation that would potentially lead to antigenic diversity in an infecting population.

From our results, the single colony strategy, commonly used in the microbiology laboratory seems to be reliable and will not lead to an underestimated measurement of the macrolide resistance rate. In the course of a single infection, horizontal gene transfer resulting in the emergence of a subset of isolates with antibiotic resistance among a susceptible population is probably rare, although we do not know if such heterogeneity is present in carriage, as it is for *S. pneumoniae* [4, 16, 21]. However, in such a large

collection it would be interesting to see if spontaneous protein size mutants are being generated and its implications for the immune system.

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CHAPTER 3

EPIDEMIOLOGICAL STUDY OF MACROLIDE-RESISTANT *S. PYOGENES* ISOLATES IN PORTUGAL (1998-2011)

Brief introduction

A study conducted in Portugal between January 1998 and June 1999 revealed a high macrolide resistance rate in *Streptococcus pyogenes* in Portugal - 35.8% [1]. The macrolide resistant population presented mostly the $cMLS_B$ phenotype (approximately 80%), while only 17% presented the M phenotype and the remaining isolates presented the $iMLS_B$ phenotype. This high macrolide resistance rate prompted continuing surveillance of antibiotic resistance, the prevalence of macrolide resistance phenotypes and genotypes and the clonal composition of the population. With this aim, a study was conducted between 1998 and 2003, including 325 macrolide resistant *S. pyogenes* isolates, associated with a diagnosis of tonsillo-pharyngitis, recovered from several laboratories located throughout Portugal. The characterization of these isolates originated two publications: the first consisted of the determination of the macrolide resistance rate, a phenotypic and a brief molecular characterization of the resistant isolates, including the prevalence of the macrolide resistance phenotypes and genotypes, as well as *emm* and T typing. The second publication included the molecular characterization of the same isolates by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). In this thesis, chapters 3.1 and 3.2 are the result of these two publications. The results of these two studies revealed that in Portugal, the overall level of macrolide resistance in *S. pyogenes* between 1998 and 2003 was high, but stable (27%), accompanied by a rapid inversion of the dominant phenotypes, with a marked decrease in the prevalence of MLS_B isolates and a concomitant increase in isolates presenting the M phenotype. The molecular characterization revealed a diverse population, with eight major PFGE clones circulating among the macrolide resistant isolates, most of them previously identified in other European countries. The high rate of macrolide resistance, as well as the rapid and unusual inversion of the macrolide resistance phenotypes motivated the continuing of the surveillance study, and the isolates recovered between 2004 and 2006 were analyzed and published in a third paper, that in this thesis corresponds to chapter 3.3. In this study, a decline in macrolide resistance was noted, due to the decrease in the prevalence of isolates presenting the M phenotype while the proportion of MLS_B isolates remained stable. The clonal composition of the population was also very diverse, with the appearance of new clones and the association of different macrolide resistant determinants to previously

identified clones. More importantly, with the inclusion of this subset of isolates in our collection it was possible to detect a decreasing trend in macrolide resistance since 1999, not previously identified, that could not be attributed to differences in the diversity of isolates presenting each of the macrolide resistance phenotypes. Macrolide consumption, known to be a major driving force in macrolide resistance, was reported to be high in Portugal, and in spite of this, erythromycin resistance reached a low value. To find out if the decreasing trend continued and to identify the circulating clones in Portugal after 2006, we conducted another study, which included macrolide resistant isolates recovered in the period 2007-2011 that in this thesis is described in chapter 3.4.

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CHAPTER 3.1

RAPID INVERSION OF THE PREVALENCES OF MACROLIDE RESISTANCE PHENOTYPES PARALLELED BY A DIVERSIFICATION OF T AND *EMM* TYPES AMONG *STREPTOCOCCUS PYOGENES* IN PORTUGAL

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SUMMARY

In Portugal, erythromycin resistance of 26.6% (n=352) remained constant during 1998 to 2003, however in 1998 the MLS_B phenotype dominated (85%), whereas in 2003 the M phenotype prevailed (77%). A decline in T12/*emm*22 MLS_B isolates could partially explain the drop in this phenotype, but the rise of the M phenotype was not due to clonal expansion.

Although penicillin remains the antibiotic of choice in the treatment of Lancefield group A streptococci (GAS) infections, macrolides and lincosamides are recommended as suitable alternatives for patients allergic to penicillin [5]. Newer macrolides, such as azithromycin, may be given once a day, making this an attractive option for the treatment of pharyngitis due to *Streptococcus pyogenes*. In Portugal, where penicillin V is not available, macrolides and lincosamides have the additional advantage of being a therapeutic option with an oral route of administration. High macrolide resistance in GAS was previously identified in Portugal [13] in line with other European countries [1, 4, 6] but in contrast to others [14, 16]. The aims of this study were to determine the prevalence of macrolide resistance phenotypes and its temporal trends and to evaluate the correlation with T and *emm*-types.

A total of 1,321 GAS from clinical infections were collected from 30 laboratories, geographically distributed throughout Portugal, from January 1998 to December 2003. The isolates were distributed in the study period as follows: 153 in 1998, 240 in 1999, 213 in 2000, 216 in 2001, 270 in 2002, and 229 in 2003. The laboratories were asked to submit all nonduplicate GAS isolated from outpatients during the study period. Antimicrobial susceptibility testing, T-typing, and macrolide resistance phenotype and genotype were determined as previously described [7, 13]. Strains were *emm* typed according to the recommendations of the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). The sequences of representatives of each restriction profile were searched against GenBank as well as the *emm* CDC database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). An isolate was considered to be of a given *emm* type if it had >95% identity over the 160 bases considered [3].

Among this collection, 352 isolates (26.6%) were erythromycin resistant. Although there was a higher prevalence of resistant isolates recovered in 1998 (34.6%), the variation of the overall prevalence in the following years (19.6% in 1999, 27.7% in 2000, 25.5% in 2001, 23.7% in 2002, and 20.5% in 2003) was not significant (χ^2 test, P = 0.22) (Fig. 1). Only the 325 erythromycin-resistant isolates recovered from throat swabs associated with a diagnosis of pharyngitis were characterized further.

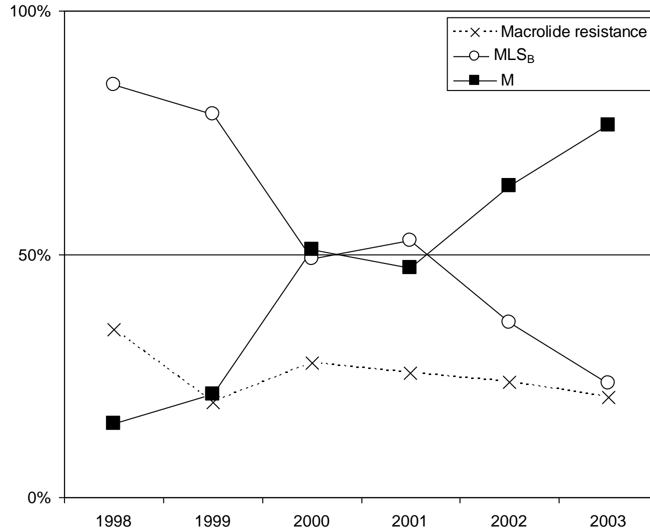

FIGURE 3.1.1 Erythromycin resistance and prevalence of macrolide resistance phenotypes in Portugal during 1998-2003

Resistance to tetracycline was expressed by 38.7% (n=126) of the isolates (MIC_{90}=96µg/ml, MIC range, 12 to 512µg/ml). The distribution of tetracycline-resistant isolates among the study years was as follows: 84.9% of the isolates recovered in 1998, 70.2% in 1999, 28.8% in 2000, 30.9% in 2001, 12.5% in 2002, and 12.8% in 2003. The $cMLS_B$ phenotype was expressed by 170 isolates (52.3%), 151 (46.4%) presented the M phenotype, and only 4 isolates (1.2%) the $iMLS_B$ phenotype. Erythromycin resistance determinants were detected in all isolates by multiplex PCR. All isolates presenting the M phenotype carried the *mef*(A) gene, including one which carried both *mef*(A) and *erm*(B) genes. Nine of the isolates presenting the MLS_B phenotype (4.9%) carried both the *mef*(A) and *erm*(B) resistance determinants whereas all other isolates presenting this phenotype yielded a single PCR product consistent with the presence of the *erm*(B) gene. Three isolates presenting the $iMLS_B$ phenotype carried the *erm*(A) gene, and one carried the *erm*(B) gene.

A summary of the number of T/*emm*-type associations with the two macrolide resistance phenotypes and their distribution over the different years of the study period is presented in Table 1. The majority (90%) of the tetracycline-resistant isolates were included in the T12/*emm*22 group.

TABLE 3.1.1 Distribution of T and *emm* types and macrolide resistance phenotypes among 325 pharyngitis associated erythromycin-resistant GAS in Portugal during 1998-2003

T types	*emm* types											**Total**
	22	12	28	1	77	75	4	9	11	2	89	
12	125/2*	2/23	4/0	1/0			0/1	0/1	2/0	0/1		**134/28**
28			29/1			0/1		0/1		0/1		**29/4**
1		0/2		0/35		0/1	0/2					**0/40**
13	3/0	0/1		0/2	3/0		0/3		1/0			**7/6**
25	1/0					0/10						**1/10**
4							0/32					**0/32**
B3264							0/2				0/2	**0/4**
5/27/44							0/2					**0/2**
14/49							0/1					**0/1**
NT	1/0	0/7	2/0	0/2			0/1					**3/10**
2		0/1					0/2			0/2		**0/5**
6						0/1	0/1					**0/2**
9								0/7				**0/7**
Total	**130/1**	**2/34**	**35/1**	**1/39**	**3/0**	**0/13**	**0/47**	**0/9**	**3/0**	**0/4**	**0/2**	**325**

* The number of strains associated to each macrolide resistant phenotype (MLS_B/M) is indicated

The situation found in 1998 and 1999, where strains expressing the MLS_B phenotype accounted for approximately 80% of all macrolide-resistant isolates (Figure 3.1.1), was similar to that found among a group of erythromycin-resistant isolates recovered in Italy in 2000 [6] and in France (2002 to 2003) [4]. However, the dominance of the MLS_B phenotype was in sharp contrast to the situation found in neighboring Spain where isolates collected around the same years (1996 to 1999) expressed overwhelmingly (95%) the M phenotype (1). The tendency for the strains expressing the MLS_B phenotype to present a limited number of T/*emm*-type associations and for at most two associations (T12/*emm*22 and T28/*emm*28) to account for more than 80% of isolates recovered in each year was a constant during the study period. The only exception was 2001 when, not only was an unusually high number of associations identified (n=9) but three different associations were necessary to account for 80% of the isolates (compare to 2000 when, for the same number of isolates, only three associations were identified) (Table 3.1.1). This indicates that other T/*emm* type associations expressing the MLS_B phenotype existed that could replace the T12/*emm*22 group; however none rose to significant numbers. In the

following years there was an increase in prevalence of isolates expressing the M phenotype, that accounted for 76.6% of erythromycin resistant isolates in 2003 (Figure 3.1.1). In sharp contrast to the data presented here, the increases in occurrence of strains expressing the M phenotype documented elsewhere were also paralleled by an increase in the overall macrolide resistance rate [8, 9, 11]. Contrary to isolates expressing the MLS_B phenotype, those expressing the M phenotype were found among a diverse group of T/*emm*-type associations and usually at least one-half of the total number of associations found each year was necessary to account for 80% of the isolates. This increased diversity is better illustrated by the strains exhibiting the M phenotype recovered in 2003, when 17 different T/*emm*-type associations were found among the 36 isolates (Table 3.1.1). Unlike these findings studies from elsewhere in Europe have documented similar numbers of T/*emm*-type associations among isolates expressing the two phenotypes [4, 6]. The contrast to the situation in Spain, where only four *emm* types accounted for 85% of the isolates expressing the M phenotype [1], indicates that, despite the geographic proximity, macrolide-resistant GAS isolated in the two countries constitute two different populations. Moreover, it suggests that the rise in isolates of the M phenotype was not due to invasion of the few *emm* groups prevalent in Spain.

The decrease observed in tetracycline resistance paralleled the decline of isolates presenting the MLS_B phenotype, namely, those associated with the T12/*emm*22 types. This could be explained by the presence of *erm* and tetracycline resistance genes in the same transposon [12] in this group of isolates.

Although a situation where either phenotype dominates is not unusual, such a rapid shift in prevalence of the different phenotypes, while maintaining the same overall erythromycin resistance rate (Figure 3.1.1), was not previously described in GAS to the best of our knowledge. This shift could not be attributed simply to the reduction in the number of isolates defined by T12/*emm*22 and the absence of other T/*emm*-type associations expressing the MLS_B phenotype that could take its place, or by the emergence of a limited number of highly successful strains expressing the M phenotype and specific T/*emm*-type associations as documented in North America [8, 11]. Since the data presented do not offer firm clues, we can only speculate as to the reasons behind this shift. Although Portugal is among the largest antibiotic consumers in Europe (www.ua.ac.be/ESAC), it is not remarkable for its macrolide consumption. Decreases in

macrolide use were associated to a reduction of macrolide resistant GAS [16]. However, there was only a slight decrease in consumption for human use in the ambulatory setting during the study period in Portugal (www.ua.ac.be/ESAC). If, as suggested by Nielsen et al. [15], tetracycline use together with macrolide use could be correlated to macrolide resistance, especially the MLS_B phenotype, then the slight reduction in tetracycline use (www.ua.ac.be/ESAC) could have an additive effect. These factors, as well as a possible immunity developed by the human population against the limited number of T antigens and M proteins associated with the MLS_B phenotype, could explain the observed reduction in prevalence. The diversity among the strains expressing the M phenotype could be explained by a higher mobility of the genetic element containing the *mef*(A) gene [10]. Interestingly, an unusual chimeric genetic element combining a transposon and a bacteriophage, that could mediate the transfer of the *mef*(A) gene by a process similar to transduction, was recently described [2]. The data presented documents a major shift in the prevalence of macrolide resistance phenotypes in an unusual short time period, not paralleled elsewhere in Europe and without a concomitant change in the frequency of macrolide resistance. Continued surveillance is needed to establish if this shift will stabilize and if the major groups of isolates sharing the same T/*emm*-types are also found elsewhere.

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CHAPTER 3.2

IDENTIFICATION OF MACROLIDE-RESISTANT CLONES OF *STREPTOCOCCUS PYOGENES* IN PORTUGAL

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SUMMARY

Although the overall level of macrolide resistance (27%) has remained stable in Portugal, a rapid inversion in the dominant phenotypes has been noted, with a sharp decrease in the MLS_B phenotype paralleled by an increase in the M phenotype. To gain further insight into these changes, 325 macrolide resistant isolates were characterized using a combination of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The use of Cfr9I, an isoschizomer of SmaI, to digest M phenotype isolates that were refractory to SmaI digestion allowed direct comparison of MLS_B and M isolates. The results from PFGE and MLST were highly concordant and identified eight major clones, accounting for 92% of the isolates, each of which was associated exclusively with a single macrolide resistance phenotype. Two major clones were found among MLS_B isolates, characterized by sequence types (ST) 46 (T12-*emm*22) and ST52 (T28-*emm*28), whereas clones characterized by ST39 (T4-*emm*4) and ST28 (T1-*emm*1) dominated among M isolates. The clone defined by ST52 corresponded to a bacitracin resistant clone circulating in Europe, and a novel variant expressing other surface antigens (T12-*emm*22) was detected. The presence of the four major clones has been reported previously in other European countries, suggesting Europe-wide dissemination of a few macrolide-resistant lineages.

INTRODUCTION

Two mechanisms of resistance to macrolide antibiotics have been described in *Streptococcus pyogenes*: one caused by the presence of a methylase encoded by the *erm*(B) or *erm*(A) genes that results in resistance to most macrolides, lincosamides and streptogramin B (the MLS_B phenotype); and a second caused by the presence of an efflux pump encoded by the *mef*(A) gene that results in resistance to 14- and 15-membered macrolides, while susceptibility to clindamycin and streptogramin B antibiotics is retained [14]. Isolates carrying both *erm* and *mef*(A) resistance determinants expressing either the MLS_B or the M phenotype have also been described previously [2].

Macrolide resistance is increasing among *S. pyogenes* isolates in several European countries, and this has often been accompanied by an alteration in the prevailing phenotypes [21]. In Portugal, macrolide resistance remained almost constant during 1998–2003 (27%) [20], but surprisingly, this was not associated with a stable population of macrolide-resistant *S. pyogenes* strains. Indeed, the predominance of the MLS_B phenotype, which accounted for c. 80% of isolates in 1998, was completely reversed in 2003, when 76.6% of the isolates expressed the M phenotype [20]. This change is puzzling, since there was no significant alteration in macrolide use during this period, and sustained use could be expected to select for isolates expressing the MLS_B phenotype, since this phenotype is associated with higher resistance *in vitro*. One possibility is that the introduction of a highly successful clone or clones, particularly from neighboring Spain, where the M phenotype predominates (95%) [1], could have displaced a local clone. However, rapid changes in *S. pyogenes* isolates associated with pharyngitis, not attributable readily to any single factor, but thought to represent natural fluctuations, have also been described previously [9].

Comparison of the profiles generated by pulsed-field gel electrophoresis (PFGE) has long been used to distinguish clones of *S. pyogenes* [3]. The endonuclease used to generate the profiles is frequently SmaI, but the emergence of isolates expressing the M phenotype with DNA that is resistant to digestion with SmaI [3] has led to the use of alternative enzymes, often SfiI, to characterize the two macrolide resistance phenotypes [10], thereby preventing a direct comparison of the M and MLS_B clones. More recently, sequence based typing methods have been developed that rely on determining the

nucleotide sequences of internal fragments of housekeeping genes [5]. These sequencing methods, known as multilocus sequence typing (MLST), provide unambiguous results that are easily portable and accessible in a central database, thereby facilitating comparison of results obtained in different laboratories.

In order to gain a better insight into the dynamic behavior exhibited by macrolide-resistant *S. pyogenes* isolates in Portugal, and to clarify the relationship of the major clones involved with those reported from other European countries [10, 18, 21], the present study characterized 325 macrolide-resistant *S. pyogenes* isolates from Portugal [20] by PFGE, using enzymes suitable for the direct comparison of M and MLS_B isolates, and MLST.

MATERIALS AND METHODS

Bacterial isolates

A collection of non-duplicate 325 macrolide-resistant *S. pyogenes* isolates recovered from throat swabs and associated with a diagnosis of tonsillo-pharyngitis between 1998 and 2003 was analyzed. The isolates, provided by 30 laboratories distributed throughout Portugal, comprised 53 from 1998, 47 from 1999, 59 from 2000, 55 from 2001, 64 from 2002, and 47 from 2003. Results of antimicrobial susceptibility tests using CLSI (formerly NCCLS) interpretative criteria [16], T-typing results, macrolide-resistant phenotypes and genotypes, and *emm* typing results, have been reported previously [20].

PFGE and MLST

Chromosomal DNA of macrolide-resistant isolates was prepared with a modification of a method described previously [7]. In brief, the composition of the lysis solution was altered to include mutanolysin 5 U/mL, lysozyme 1 mg/mL and ribonuclease A 30 µg/mL (Sigma-Aldrich, Steinheim, Germany). After digestion with SmaI or Cfr9I (Fermentas, Vilnius, Lithuania), the fragments were resolved by PFGE as described previously [7]. All MLS_B isolates produced multiple bands following digestion with SmaI; however, the DNA of most isolates with the M phenotype (n = 150; 46.2%) did not digest with SmaI, and in such cases the isoschizomer Cfr9I (Fermentas) was used. Bionumerics software (Applied-Maths, Sint-Martens- Latem, Belgium) was used to create UPGMA (unweighted pairgroup method with arithmetic mean) dendrograms of the SmaI or Cfr9I fragment patterns. The Dice similarity coefficient was used, with optimization and position tolerance settings of 1.0 and 1.5, respectively. PFGE clusters were defined as isolates with ≥ 80% similarity, as described previously for *Streptococcus pneumoniae* [19]. A PFGE-based cluster was considered to be a major lineage if it included ten or more isolates, or if it included five or more isolates recovered in the same year. MLST analysis was performed with representatives of each major lineage, as described previously [5], and allele and sequence type (ST) identification were performed using the *S. pyogenes* MLST database (http://spyogenes.mlst.net).

Bacitracin susceptibility testing

Bacitracin susceptibility was determined for all isolates by disk diffusion, using disks containing bacitracin 0.05 U (Oxoid, Basingstoke, UK) and Tryptic Soy Agar (Oxoid), supplemented with sheep blood 5% v/v, according to the manufacturer's instructions. Any zone of inhibition around the bacitracin disk was interpreted as being indicative of susceptibility.

RESULTS

PFGE and MLST

All 325 isolates included in this study were typeable by PFGE, and 40 (12.3%) isolates were analyzed by MLST. Eight major lineages were found, containing 297 (91.4%) of the isolates. The PFGE patterns of representative isolates from each major clone are shown in Figure 3.2.1. The remaining isolates were included in minor PFGE groups (containing six or fewer isolates) or had unique PFGE profiles. In most cases, all isolates belonging to the same PFGE-defined cluster had the same ST by MLST. For the two exceptions, the STs were single-locus variants of each other, indicating a close genetic relationship between the isolates. Similarly, most isolates belonging to the same PFGE cluster had the same *emm* type and the same resistance phenotype and genotype. The characteristics of the eight lineages found in this study, as well as their distribution during the study period, are summarized in Table 3.2.1.

TABLE 3.2.1. Properties of macrolide-resistant *Streptococcus pyogenes* responsible for pharyngitis isolated in Portugal during 1998-2003

PFGE	No. of isolates (%)	T/*emm*	Phenotype	Genotype	ST	Year					
						1998	1999	2000	2001	2002	2003
A	118 (36.3)	12/22[a]	MLS_B	*erm*(B)[b]	46[c]	44	32	17	13	7	5
B	46 (14.2)	28/28[d]	MLS_B	*erm*(B)[e]	52	0	2	12	12	14	6
C	48 (14.8)	4/4[f]	M	*mef*(A)	39[g]	5	3	7	3	10	20
D	20 (6.2)	12/12[h]	M	*mef*(A)	36	1	4	3	5	5	2
E	10 (3.1)	12/12	M	*mef*(A)	36	0	2	3	1	2	2
F	35 (10.8)	1/1[i]	M	*mef*(A)	28	0	1	5	7	16	6
G	12 (3.7)	25/75	M	*mef*(A)	150	0	0	0	3	5	4
H	8 (2.5)	9/9	M	*mef*(A)	75	0	0	6	0	2	0
Other[j]	28 (8.6)					3	3	6	11	3	2

[a] 13/22 (n=3), 25/22 (n=1)
[b] All the isolates presented the MLS_B, phenotype, and 111 isolates expressed the *erm*(B) gene, while 7 isolates carried both *erm*(B) and *mef*(A).
[c] ST45 (n=1)
[d] 12/22 (n=9)
[e] All the isolates presented the MLS_B, phenotype, and 45 isolates expressed the *erm*(B) gene, while only one isolate carried both *erm*(B) and *mef*(A)
[f] Seven other T types were found, (T5/27/44, T6, T1, TB3264, T12, T13 and T25), including one isolate presenting T12/*emm*22.
[g] ST38 (n=1)
[h] 13/12 (n=1); 2/12 (n=1)
[i] 13/1 (n=1)
[j] Fifteen T/*emm* type combinations were found among 13 PFGE clusters

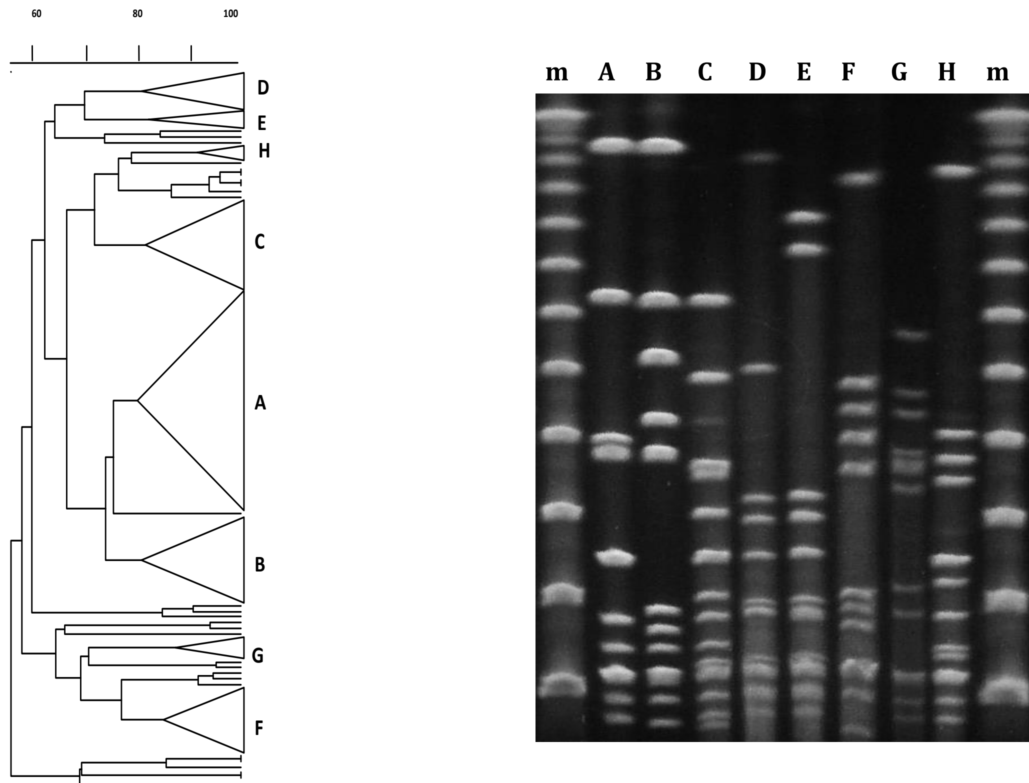

FIGURE 3.2.1 Pulsed-field gel electrophoresis (PFGE) analysis of macrolide-resistant *Streptococcus pyogenes* isolates from Portugal. (A) Dendrogram showing cluster analysis of the PFGE profiles of the 325 macrolide-resistant isolates by the unweighted pair-group with arithmetic mean (UPGMA) method. For each of the major clones, a triangle proportional to the number of isolates is shown in the dendrogram, followed by the capital letter designating the PFGE cluster. (B) PFGE profiles generated following SmaI (A, B) or Cfr9I (C–H) digestion of DNA isolated from representatives of each major clone. Capital letters identifying each lane correspond to the clone designations

The largest cluster (cluster A; 118 (36.3%) isolates) comprised exclusively MLS_B isolates. MLST was performed for nine of these isolates, and all except one belonged to ST46, with the remaining isolate belonging to ST45, which is a single-locus variant. This cluster also included most (n=7) of the isolates that carried the *erm*(B) and *mef*(A) resistance determinants simultaneously. These seven isolates were recovered from two hospitals in the same region during1998 and 1999.

Cluster B accounted for 46 (14.2%) of the isolates (all MLS_B), most of which were T28/*emm*28. However, a significant proportion (n=9; 19.6%) of the isolates in this cluster had a different T and *emm* type (T12-*emm*22). Ten (21.8%) of these 46 isolates, representing both T-*emm* combinations present in this cluster, were analyzed by MLST.

All ten isolates characterized by MLST, defined either by *emm*22 or *emm*28, belonged to ST52.

The most heterogeneous cluster found in this study (cluster C) accounted for 48 (14.8%) of the isolates, all of which expressed the M phenotype. Although almost all of these isolates were *emm*4, the T types present in this cluster were very diverse. The dominant T type was T4, but other T types, such as 5/27/44, T6, T1, B3264, T12, T13 and T25, accounted for 34.8% of the isolates in this cluster. Despite this diversity, three of the four isolates analyzed by MLST were ST39; the exception, a T12-*emm*4 isolate, belonged to ST38. However, these STs are single-locus variants of each other, supporting the overall grouping revealed by PFGE analysis. Isolates presenting with *emm*12 were divided into two PFGE clusters (clusters D and E; Figure 3.2.1), but this distinction was not supported by the other methods used. All the isolates in these two clusters expressed the M phenotype, and almost all had the same T type (T12). Moreover, a single ST (ST36) was associated with both clusters, suggesting that clusters D and E represent subgroups of the same genetic lineage, together accounting for 30 (9%) of the isolates.

Cluster F (n = 35; 10.8%) included *emm*1 isolates, all of which expressed the M phenotype. The four isolates from this cluster that were analyzed by MLST included one with a T type different from T1, namely T13, but all were found to belong to ST28. Two smaller PFGE clusters (clusters G and H) included 12 and eight isolates, respectively (Table 3.2.1).

A small proportion (n = 28; 8.6%) of isolates did not belong to any of the clusters described above. Five had a unique PFGE pattern, and although the *emm* types of most (n=4) of these were represented in the major clones (i.e., *emm*1, 9, 22 and 75), these isolates had diverse genetic backgrounds.

Bacitracin susceptibility

All isolates grouped in PFGE cluster B were resistant to bacitracin, as shown by the absence of an inhibition zone around the bacitracin disk, whereas the remaining 279 isolates were bacitracin- susceptible.

DISCUSSION

Although the overall level of erythromycin resistance remained constant during the study period, the MIC_{50} for the isolates recovered during 1998 was >512 mg/L, compared with 16 mg/L in 2003, suggesting that changes had occurred in the clonal composition of the macrolide-resistant population. The strains analyzed represented diverse genetic lineages corresponding to 21 PFGE profiles. A large diversity among macrolide- resistant isolates, notably among isolates presenting the M phenotype, is not unusual [4, 22]. Nevertheless, eight clones accounted for most (92%) of the isolates, and the four most frequent lineages (PFGE clusters A, B, C and F) represented 76% of the isolates analyzed. Despite significant yearly variations, six of the major clones identified were detected during five of the six study years, indicating their persistence in the population. Also noteworthy, and in agreement with previous studies, was the strong association between *emm* type and ST [5], as well as between PFGE cluster and macrolide resistance phenotype. Although the latter association has been reported previously [24], these comparisons were frequently incomplete because of the existence of isolates expressing the M phenotype that were not digested by SmaI, which is the endonuclease used most frequently for PFGE analysis [10]. The data reported here confirm the above findings for all isolates, since use of the isoschizomer Cfr9I allowed direct comparison of all M and MLS_B isolates. Most of the isolates grouped in each PFGE cluster also shared the same *emm* type, with the notable exception of cluster B, in which a significant fraction (n=9, 19.6%) were *emm*22. Although these isolates did not form a PFGE subgroup within cluster B, they also shared a T type (T12) that was different from that of other isolates in cluster B, and were indistinguishable, according to these surface markers, from clone A. However, MLST analysis confirmed the inclusion of these isolates in clone B, since the T28-*emm*28 and T12-*emm*22 isolates shared ST52, having no alleles in common with ST46, which is characteristic of clone A. Moreover, all these isolates shared the unusual property of being resistant to bacitracin, a test used traditionally in the presumptive identification of *S. pyogenes* [23]. The first outbreak involving macrolide-resistant (MLS_B) *S. pyogenes* isolates resistant to bacitracin was described in the USA [23], but no data regarding T and *emm* type are available. Recently, macrolide- and bacitracin-resistant *S. pyogenes* isolates with T28-*emm*28 have been described in Europe [11, 15], including Spain, where these

isolates were associated with ST52 [17], the same ST of the isolates found in Portugal. A report from Sweden [6] also identified T28-*emm*28 isolates belonging to ST52 as the dominant clone among invasive isolates; however, the macrolide and bacitracin susceptibility of the isolates involved was not reported. The variant reported in the present study, sharing the same surface antigens as the dominant MLS_B clone T12-*emm*22, has not been reported previously, and perhaps resulted from transfer of the necessary genetic determinants into the ST52 background, as has been suggested to account for the presence of multiple *emm* types associated with the same ST [9]. The substantial temporal variations in clonal prevalence among macrolide-resistant *S. pyogenes*, as documented in Portugal [20], highlight the pitfalls of comparing the data from various studies conducted in Europe. Moreover, there are few studies that have characterized the genetic backgrounds of macrolide-resistant *S. pyogenes* by MLST, and studies using PFGE frequently resort to different endonucleases (usually SfiI) to characterize M phenotype isolates. Further complicating the comparison among studies is the inclusion of isolates responsible for different types of infection, since an association between *emm* type and particular disease manifestations [13] could create a bias towards different *emm* types. Despite these limitations, three surveys of STs among macrolide-resistant *S. pyogenes* in European countries with a lower rate of macrolide resistance than Portugal identified similar clones. Thus, in a survey in Germany, in which isolates with the M phenotype predominated (n=30), the most common clone was ST39-*emm*4, with other important clones including ST28-*emm*1 and ST36-*emm*12 [18], i.e., similar to the present findings in Portugal. In Poland, ST36-*emm*12 accounted for three of five isolates expressing the M phenotype [21]. However, in both Germany and Poland, isolates expressing the MLS_B phenotype carried the *erm*(A) gene, unlike the situation in Portugal, and the few isolates carrying the *erm*(B) gene were unrelated to the major clones identified in Portugal. It is noteworthy that three of the four isolates from Portugal with an inducible MLS_B phenotype shared the same *emm* gene as one of the dominant clones expressing this phenotype in both Poland and Germany (ST63-*emm*77). A strong parallel between the data reported here and a study of macrolide-resistant *S. pyogenes* isolates recovered in Belgium during a similar period was also noted. Although only data concerning PFGE clustering, *emm* type and macrolide resistance phenotype and genotype are available for comparison, the major clones identified in Belgium seem to correspond to those found in Portugal. Two PFGE clusters, containing

*emm*22 and *emm*28 isolates, respectively, accounted for the majority of *erm*(B) isolates, and two PFGE clusters, containing *emm*1 and *emm*4 isolates, respectively, accounted for the majority of *mef*(A) isolates [10]. This strong similarity existed despite an overall lower level of macrolide resistance in Belgium and the temporal variations in the prevalence of the different phenotypes compared with Portugal [10, 20]. These observations argue in favor of Europe wide dissemination of a limited number of macrolide-resistant clones, although each country may retain specificity regarding the prevalence of the different genotypes and clones. It is currently unclear whether these resistant clones are spreading throughout Europe, or whether they result from independent acquisition of resistance genes by the same prevalent STs, followed by local dissemination. Whenever these clones account for the majority of macrolide-resistant isolates, they frequently share the same resistance determinants, regardless of the country of origin, suggesting geographical dissemination. Nevertheless, local acquisition of resistance genes must also play a role, since isolates fully susceptible to macrolides that share the same STs as these major clones have been described [12], and either of the resistant determinants has been associated with each lineage, both in the present report and in previous studies [18, 21]. This hypothesis is further supported by previous observations showing that conjugal transfer of elements carrying either the *erm* or the *mef*(A) resistance determinants among *S. pyogenes* can occur *in vitro* [8]. The alterations in the prevalence of macrolide resistance differ significantly among different European countries and cannot be correlated directly with use of macrolides [10, 20]. As expected, a situation in which macrolide resistance is changing can be accompanied by changes in the prevalent clones [10], but such changes can also occur when macrolide resistance is stable [20], suggesting that other factors, such as natural fluctuations in the prevalence of clones [9], may be important determinants of macrolide resistance and the associated phenotypes.

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CHAPTER 3.3

DECREASE IN MACROLIDE RESISTANCE AND CLONAL INSTABILITY AMONG *STREPTOCOCCUS PYOGENES* IN PORTUGAL

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SUMMARY

Macrolide resistance among *Streptococcus pyogenes* (group A streptococci) in Portugal was stable during 1998–2003, but a rapid inversion in the dominant phenotypes was noted in the same period, with a sharp decrease in the proportion of isolates presenting the MLS_B phenotype and a concomitant increase in isolates presenting the M phenotype. The characterization of group A streptococci recovered during 2004–2006, which is reported here, revealed that resistance was not stable during this period and that the decline in erythromycin resistance observed during 2004–2006 was due to a decrease in the prevalence of isolates presenting the M phenotype, while the proportion of isolates expressing the MLS_B phenotype remained stable. Characterization by *emm* typing, T serotyping, pulsed-field gel electrophoresis (PFGE) profiling and multilocus sequence typing revealed a very diverse population. Several of the major PFGE clusters identified had already been found in the 1998–2003 study period, but others were found for the first time, e.g. T11-*emm*11-ST403, carrying the *erm*(B) gene, and T3/13-*emm*3-ST315, carrying the *mef*(A) gene. The clone defined as T12-*emm*12-ST36, previously found to be associated with *mef*(A), was now found to be predominantly associated with *erm*(B). The clonal dynamics of macrolide-resistant group A streptococci emphasizes the importance of considering factors other than antibiotic consumption in explaining the prevalence of resistant isolates.

INTRODUCTION

Streptococcus pyogenes, Lancefield group A streptococci (GAS), is an important human pathogen causing a wide variety of infections, from severe life-threatening diseases to pharyngitis, an infection for which it is the most common bacterial etiological agent. β-lactams remain the antibiotics of choice in the treatment of GAS pharyngitis, and macrolides and lincosamides are the first line alternatives. Although macrolide resistance in GAS remained at low levels for a long time, a number of recent studies have reported an increase in resistance. Two different mechanisms have been recognized in macrolide-resistant *S. pyogenes*: target site modification and active efflux. Target site modification occurs in the ribosome via an erythromycin resistance methylase (Erm) protein, blocking the binding of macrolides, lincosamides and streptogramin B (generating the MLS_B phenotype) [19]. In GAS, the MLS_B phenotype can be mediated by two classes of methylase genes, the *erm*(B) determinant and the *erm*(TR) determinant (belonging to the *erm*(A) class) [27]. The expression of the *erm* genes can be either constitutive or inducible, generating the $cMLS_B$ phenotype or the $iMLS_B$ phenotype, respectively, the latter frequently being associated with the *erm*(A) class. Both classes of *erm* genes were found to be associated with transposons that were shown to have the capacity to transfer the resistance traits to susceptible isolates by conjugation [8]. The second mechanism conferring macrolide resistance in GAS is mediated by a membrane-associated pump encoded by the *mef* genes, leading to resistance to 14- and 15-membered ring macrolides (generating the M phenotype) [28]. The *mef*(A) and *mef*(E) variants are widely distributed in streptococci [4], although the *mef*(A) variant associated with a phage-like element was found in the majority of GAS with the M phenotype [15]. Other mechanisms of resistance resulting from mutations, such as alterations of the ribosomal proteins, are infrequently observed in isolates responsible for infections, and currently have little clinical impact [7].

The factor most frequently associated with increases in antimicrobial resistance is antimicrobial consumption [1, 6]. An association between macrolide consumption and resistance in GAS was shown in ecological studies [6, 17], with intermediate-acting (e.g. clarithromycin) and, particularly, long-acting (e.g. azithromycin) macrolides being implicated in enhanced resistance selection [11]. Further supporting this association, it was noted that a sharp decrease in macrolide prescribing was accompanied by a decline in

macrolide-resistant GAS [29]. More recently, studies at the individual level confirmed and extended these findings by showing a causal relationship between both clarithromycin and azithromycin treatment and selection for macrolide-resistant streptococci [21]. Furthermore, the latter study implicated clarithromycin, but not azithromycin, in the selection for the *erm*(B) gene. In spite of the recognized importance of antibiotic consumption in the selection of resistant strains, the largely clonal structure of most bacterial populations [22], including GAS [16, 22], suggests that the circulating clones may also contribute significantly to both the prevalence of resistance phenotypes and the overall level of resistance. The transmissibility of the genetic elements carrying the resistance determinants may also influence their prevalence in the population, with the more easily disseminated elements having an advantage over less mobile genetic determinants [20].

We have previously shown that, although erythromycin resistance in GAS remained above 20% in Portugal from 1998 to 2003, this was not associated with a stable population of macrolide resistant S. pyogenes [31]. Indeed, the predominance of the MLS_B phenotype, which accounted for c. 80% of isolates in 1998, was completely reversed in 2003, when almost 77% of the isolates expressed the M phenotype [31]. We have also found these changes to be associated with the decline of a particular clone expressing the MLS_B phenotype and the emergence of several clones expressing the M phenotype [30]. Here we report the continuing fluctuations in macrolide resistance phenotypes and a decline in overall erythromycin resistance.

MATERIALS AND METHODS

Bacterial isolates and identification

In total, 1184 *S. pyogenes* isolates recovered from throat swabs and associated with a diagnosis of tonsillo-pharyngitis were collected from 31 microbiology laboratories located throughout Portugal from January 2004 to December 2006. The laboratories were asked to submit all non-duplicate *S. pyogenes* isolates from outpatients during the study period. The isolates were collected in the study period as follows: 284 in 2004, 392 in 2005, and 508 in 2006; only a little over 5% of the isolates were recovered from adults (>18 years). The proportion of isolates submitted by laboratories from each of the major regions of Portugal was constant relative to the previous study period [31]. Isolates were identified to the species level by colony morphology, β-haemolysis on horse blood agar, and a commercial latex agglutination technique (Slidex Strepto A; BioMérieux, Marcy l'Etoile, France). In this collection, 156 isolates (13.2%) were erythromycin-resistant, and only these isolates were characterized further.

Antimicrobial susceptibility testing and macrolide resistance phenotype

Susceptibility to erythromycin, clindamycin and tetracycline (Oxoid, Basingstoke, UK) was tested using disk diffusion according to CLSI recommendations [10]. The macrolide resistance phenotype was determined according to a double disk test previously described [23].

Bacitracin susceptibility was determined for all isolates by disk diffusion using disks containing 0.05 U of bacitracin (Oxoid, Basingstoke, UK) as previously described [30]. The absence of an inhibition zone around the disk was interpreted as resistance.

PCR determination of the macrolide and tetracycline resistance genotype

Total bacterial DNA was isolated according to the methodology described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). PCR reactions to determine which of the macrolide resistance determinants (*erm*(B), *erm*(A) or *mef*) was present were performed as described previously [14]. To discriminate between *mef*(A) and *mef*(E), mef was amplified by PCR using primers MEFR (5'-CCAATGA TTTACACCGATT-3'), MEF1 (5'-AATACAACAATTGGAAACTT-3') and MEF2 (5'-

AAGGAGTTGTGGTTCTGA-3'), as previously described (Gómez E, de la Pedrosa G, van derLinden M *et al*. Presented at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Chicago, 17–20 September 2007).

PCR reactions for the detection of the tetracycline resistance determinants *tet*(K), *tet*(L), *tet*(M) and *tet*(O) were performed for all isolates included in this study, as previously described [32]. The *Streptococus dysgalactiae* subsp. *equisimilis* strains SH533, SH523 and 645040, carrying *tet*(M), *tet*(O) and *tet*(L), respectively, and an *Escherichia coli* strain carrying the pST181 plasmid with the *tet*(K) gene were used as positive controls.

T and *emm* typing

T typing was done by slide agglutination using sera for types 1, 2, 3, 4, 6, 8, 9, 11, 12, 13, 18, 22, 23, 25, 28, 5 ⁄ 27 ⁄ 44, 14 ⁄ 19, B3264 and Imp. 19 (Hemolytic streptococcus Typing Antisera for Group A (T-typing), Seiken, Denka Seiken, Tokyo, Japan), according to the manufacturer's instructions. *emm* typing was performed as described by the CDC (http:// www.cdc.gov/ncidod/biotech/strep/protocols.htm). Amplification products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions, and sequenced using primer emmseq2 [5], and the DNA sequences were searched against the *emm* sequences deposited in the CDC database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). An isolate was considered to be of a given *emm* type if it had >95%identity over the 160 bases considered [5].

Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

PFGE was performed as previously described [9]. All isolates were digested with SmaI, and the isoschizomer Cfr9I was used only for the isolates with the M phenotype, which were not digested by SmaI, due to the presence of a methyltransferase encoded in the same genetic element that carries the *mef*(A) gene [15, 30]. Bionumerics software (Applied-Maths, Sint-Martens- Latem, Belgium) was used to create UPGMA (unweighted pair-group method with arithmetic mean) of the SmaI- or Cfr9I- generated fragment patterns. The Dice similarity coefficient was used, with optimization and position tolerance

settings of 1.0 and 1.5, respectively. PFGE clusters were defined as isolates with ≥ 80% similarity [9]. A PFGE-based cluster was considered to be a major lineage if it included more than five isolates. MLST analysis was performed in representatives of each major lineage, as previously described [12], and allele and sequence type (ST) were attributed using the *S. pyogenes* MLST database (spyogenes.mlst.net).

Statistical analysis

Wallace coefficients (W) were used to compare partitions. This coefficient indicates the probability that two isolates sharing the same characteristic, as established using a given typing method, will also be grouped together when a different typing method is used [9]. Simpson's index of diversity (SID) and corresponding 95% CIs were used to evaluate the clonal diversity of the isolates presenting the M and the MLS_B phenotypes [9]. Statistically significant differences in proportions of resistant isolates were detected using the two-tailed Fisher exact test, trends in macrolide resistance were evaluated using the Cochran–Armitage test for trend [2], and 95% CIs were calculated using the Wilson method [3].

RESULTS

Antimicrobial susceptibility

During the 3 years of the study, the overall rate of erythromycin resistance was 13.2%, lower than that documented previously (26.6%, Fisher's exact test, $p < 10^{-6}$) [31]. In 2004, 50 isolates were erythromycin-resistant (17.6%), the highest resistance rate, followed by 11% (n=43) in 2005 and 12.4% (n=63) in 2006, with a significant decreasing trend (Cochran-Armitage test for trend, $p < 10^{-4}$). Inspection of Figure 3.3.1 shows that the decline in resistance in the 2004-2006 period was due to a decline in isolates of the M phenotype, and that this trend of decreasing erythromycin resistance could have started before 2004. In fact, the Cochran-Armitage test for trend, which was unable to detect a trend in the period 1999–2003 (p = 0.22), is significant if one considers the entire period 1999–2006 ($p < 10^{-4}$).

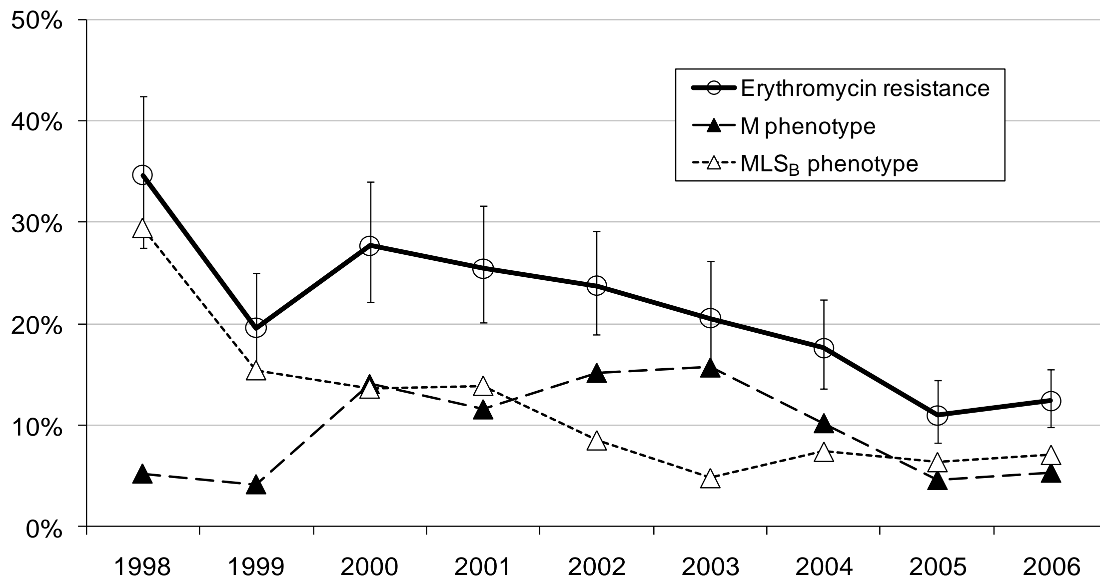

FIGURE 3.3.1. Erythromycin resistance and prevalence of macrolide resistance phenotypes in Portugal during the period 1998–2006. Open circles and solid lines represent the proportion of erythromycin-resistant group A streptococci among those causing pharyngitis and corresponding 95% CIs. Broken lines represent the proportion of each phenotype in the population. Solid triangles represent the proportion of isolates of the M phenotype. Open triangles represent the proportion of isolates of the MLS_B phenotype.

The majority of the isolates (n=80; 51.3%) presented the $cMLS_B$ phenotype, 74 (47.4%) presented the M phenotype, and only two isolates (1.3%) presented the $iMLS_B$

phenotype; however, the prevalence of the macrolide-resistance phenotypes was not constant during the study period (Figure 3.3.1). Resistance to tetracycline was found in 19.9% (n = 31) of the macrolide-resistant isolates, all expressing the MLS_B phenotype; in fact, the macrolide resistance phenotype was a good predictor of tetracycline resistance (W=0.739). The distribution of tetracycline-resistant isolates during the study years was as follows: 20.0% (n=10) of the isolates recovered in 2004, 11.6% (n=5) in 2005, and 25.4% (n=16) in 2006.

Resistance to bacitracin was found in 43 isolates, all presenting the $cMLS_B$ phenotype.

Genotypic characterization

Erythromycin resistance determinants were detected in all isolates using multiplex PCR. With the exception of seven isolates, all carried a single macrolide resistance determinant (Table 3.3.1). All isolates of the M phenotype carried *mef*(A) and none the *mef*(E) variant, including two that carried both *mef*(A) and *erm*(B) and an isolate that carried *mef*(A) and *erm*(A). Four of the isolates presenting the $cMLS_B$ phenotype (4.9%) carried both *erm*(B) and *mef*(A), whereas all other isolates presenting this phenotype yielded a single PCR product consistent with the presence of *erm*(B). One of the two isolates presenting the $iMLS_B$ phenotype carried *erm*(A) and the other *erm*(B). The *tet*(M) gene was found among all tetracycline resistant isolates. None of the other *tet* determinants was found among the studied isolates, including all phenotypically tetracycline susceptible isolates.

Clonal characterization

The *emm* types most frequently found in this study were *emm*28 (28%), *emm*4 (25%) and *emm*11 (11%). Other *emm* types accounted for 37% of the isolates, but each for less than 10% (*emm*1, *emm*2, *emm*3, *emm*4, *emm*6, *emm*12, *emm*22, *emm*44–61 and *emm*75). The most prevalent T types were T28 (25%), T4 (21%), T12 (17%) and T3/13 (12%), and the remaining 24% of the isolates were T1, T6, T11, T25 or were T non-typeable.

All 156 isolates were typeable by PFGE using either SmaI or Cfr9I, and 25 (16%) of the isolates were analyzed using MLST. Eight major lineages were identified,

containing 139 isolates (89%), and the remaining isolates (n=17) were included in minor PFGE clusters (containing four or fewer isolates) or had unique PFGE patterns. The PFGE patterns of representative isolates from each major clone are shown in Figure 3.3.2 and the characteristics of the eight major lineages found in this study, as well as their distribution during the study period, are summarized in Table 3.3.1.

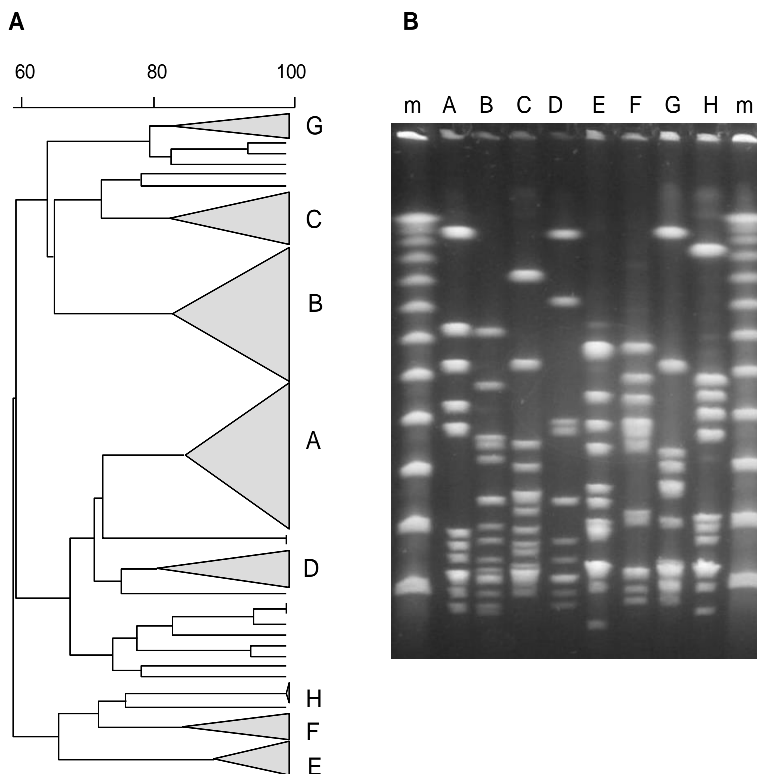

FIGURE 3.3.2 Pulsed-field gel electrophoresis (PFGE) analysis of macrolide-resistant *Streptococcus pyogenes* isolates from Portugal. (a) Dendrogram showing cluster analysis of the PFGE profiles of the 156 macrolide-resistant isolates by the unweighted pair-group with arithmetic mean (UPGMA) method. Dice coefficients (percentages) are indicated in the scale above the dendrogram. For each of the major clones, a triangle proportional to the number of isolates is shown in the dendrogram, followed by the capital letter designating the PFGE cluster. (b) PFGE profiles generated following SmaI (MLSB isolates) or Cfr9I (M isolates) digestion of DNA isolated from representatives of each major clone. Capital letters identifying each lane correspond to the clone designations. m, lambda ladder PFGE marker (New England Biolabs, Beverly, MA, USA).

TABLE 3.3.1. Properties of macrolide-resistant *Streptococcus pyogenes* responsible for pharyngitis isolated in Portugal during the period 2004–2006

PFGE cluster	No. of isolates	T/*emm*	Phenotype	Genotype	ST	Year		
						2004	2005	2006
A	42 (26.9)	28/28[a]	$cMLS_B$	*erm*(B)[b]	52	10	17	15
B	39 (25)	4/4[c]	M	*mef*(A)[d]	39	18	14	7
C	16 (10.3)	11/11[e]	$cMLS_B$	*erm*(B)	403	4	3	9
D	11 (7.1)	12/22	$cMLS_B$	*erm*(B)[f]	46	5	1	5
E	10 (6.4)	3/13/3	M	*mef*(A)	315	0	0	10
F	8 (5.1)	25/75	M	*mef*(A)	150	3	1	4
G	7 (4.5)	12/12	$cMLS_B$[g]	*erm*(B)[g]	36	1	2	4
H	6 (3.8)	1/1	M	*mef*(A)[h]	28	3	3	0
Other[i]	17 (10.9)					6	2	9

PFGE, pulsed-field gel electrophoresis; ST, sequence type.
[a]NT/28 (n = 2); 12/28 (n = 2).
[b]Two isolates carried both *erm*(B) and *mef*(A).
[c]3/13/4 (n = 5); 3/13/2 (n = 1); 3/13/3 (n = 1).
[d]One isolate carried both *erm*(B) and *mef*(A) and one isolate carried both *mef*(A) and *erm*(A).
[e]12/11 (n = 3).
[f]Two isolates carried both *erm*(B) and *mef*(A).
[g]One isolate had the $iMLS_B$ phenotype and carried *erm*(A).
[h]One isolate carried both *mef*(A) and *erm*(A).
[i]Twelve T/*emm* type combinations were distributed among ten PFGE clusters. All three macrolide resistance phenotypes were found among these isolates: M phenotype (n = 10), all carrying the *mef*(A) gene; $cMLS_B$ phenotype (n = 6), all carrying the *erm*(B) gene and $iMLS_B$ phenotype (n = 1), carrying the *erm*(B) gene.

The PFGE cluster classification was an excellent predictor of both the macrolide resistance phenotype and the *emm* type (W=0.995 and W=0.958, respectively).

PFGE cluster A accounted for 27% (n=42) of the isolates and included exclusively the $cMLS_B$ phenotype. All isolates in this cluster carried the *emm*28 allele and the *erm*(B) gene, including two isolates that additionally carried *mef*(A), and all were susceptible to tetracycline and, unusually, resistant to bacitracin. Most isolates in this cluster were T28, with the exception of two isolates that were non-typeable and two isolates that were T12. All the isolates characterized by MLST (n=4), including one with the T12 serotype, belonged to ST52. A single bacitracin-resistant isolate was not grouped into any of the major PFGE clusters but had the same characteristics as these isolates (T28-*emm*28-ST52).

Cluster B included only isolates of the M phenotype and they were susceptible to tetracycline, and accounted for 25% (n=39) of the isolates. All the isolates carried *mef*(A),

including two isolates that, in addition, also carried either *erm*(B) or *erm*(A). Although almost all the isolates were T4, a significant proportion (n=7; 17.9%) of the isolates were T3/13. However, only two of these seven isolates were of different *emm* types, namely *emm*2 and *emm*3, whereas all other isolates of the cluster carried *emm*4. All isolates analyzed by MLST (n=6), including the two isolates of different *emm* types, were found to belong to ST39.

All the isolates included in cluster C (n=16, 10.3%) presented the $cMLS_B$ phenotype, were resistant to tetracycline, and carried *erm*(B). Most isolates included in this cluster were T11-*emm*11, with the exception of three isolates, characterized by the combination T12-*emm*11. All isolates of this PFGE cluster for which the sequence type was determined (n=4), including one T12-*emm*11 isolate, belonged to ST403.

Cluster D comprised 11 $cMLS_B$ isolates (7.1%), which were resistant to tetracycline and had the same combination of surface antigens, namely T12-*emm*22. Two isolates carried both *erm*(B) and *mef*(A), and those remaining carried only *erm*(B). The three isolates included in this PFGE cluster analyzed by MLST were found to belong to ST46.

All of the isolates grouped in cluster E (n=10, 6.4%) presented the M phenotype, were susceptible to tetracycline, and carried only *mef*(A). The T antigen expressed by all of the isolates was T3/13, and all of them carried the *emm*3 allele. MLST was performed for two isolates, and both belonged to ST315.

Three smaller clusters (F, G and H) comprised eight, seven and six isolates, respectively. Cluster F included tetracycline-susceptible isolates displaying the M phenotype and the T25-*emm*75 surface antigens and carrying *mef*(A). One isolate was analyzed by MLST and belonged to ST150. Cluster G comprised tetracycline-susceptible $cMLS_B$ isolates, carrying *erm*(B), with the exception of one isolate that presented the $iMLS_B$ phenotype and carried *erm*(A). All seven isolates were T12-*emm*12, and the two isolates analyzed by MLST were ST36. The smaller cluster, designated H, included tetracycline-susceptible isolates presenting the M phenotype, and all except one carried only *mef*(A) (the exceptional isolate carried both *mef*(A) and *erm*(A)). All of the isolates were T1-*emm*1, and the ST found in this cluster was ST28 (n=2).

A small proportion (n=17, 10.9%) of the isolates was not grouped into any of the clusters described above. Whereas six had a unique PFGE pattern, the *emm* types of another six were found in major clusters (*emm*1, *emm*12 and *emm*3). The prevalence of the

major clusters was not stable during the study period (Table 3.3.1), and these fluctuations were reflected in the changes observed in the frequency of the macrolide resistance phenotypes (Figure 3.3.1). To evaluate whether the diversity of the clones expressing each of the main macrolide resistance phenotypes— MLS_B and M—varied during the period 1998–2006, SID and the corresponding 95% CIs were calculated for each year. The results are summarized in Figure 3.3.3.

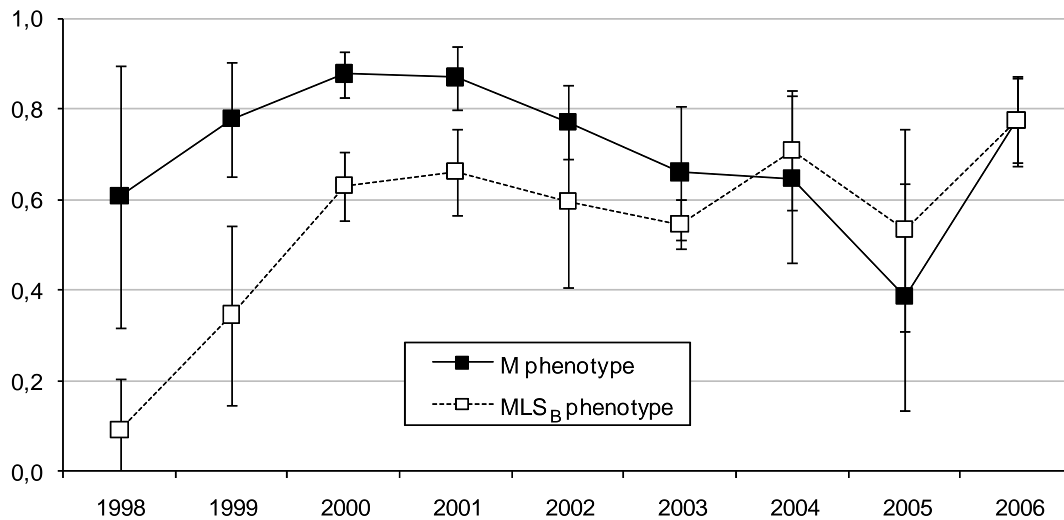

FIGURE 3.3.3 Simpson's index of diversity for the population of macrolide-resistant group A streptococcus (1998–2006). Simpson's index of diversity and corresponding 95% CIs were calculated for the PFGE clusters of macrolide-resistant *Streptococcus pyogenes* isolates from each study year.

DISCUSSION

The erythromycin resistance rate among GAS during the period 2004–2006 decreased significantly, and included the lowest value found during the last decade in Portugal. Excluding 1998, when the overwhelming dominance of a single clone could have influenced the overall rate of resistance to erythromycin [30], a re-evaluation of the trend in the period 1999–2006 revealed a significant decrease in the entire period, contrary to our previous findings for the period 1999–2003 [31]. This steady decrease in resistance, accompanied by changes in the prevalence of the macrolide resistance phenotypes, was due to a clonal instability that continued during the period reported here. The emergence of clones not detected previously, such as the PFGE clusters characterized by T11-*emm*11-ST403 and T3/13-*emm*3-ST315, accounting for a large fraction of resistant isolates, was accompanied by a change in the prevalence of the clones previously described and even the disappearance of some clones. Most clusters retained the same macrolide resistance determinant found in the period 1998–2003, apart from the unusual PFGE cluster characterized by T12-*emm*12-ST36, which was now exclusively associated with the MLS_B phenotype and the *erm*(B) gene, instead of with the M phenotype detected in the period 1999-2003. With the exception of a macrolide-resistant clone not previously described (T3/13-*emm*3-ST315), all clones identified among macrolide-resistant GAS in Portugal appear to be present in other European countries, suggesting wide geographical spread of a few successful clones [13, 18, 24, 25, 28]. However, the diversity of the clones detected in a single geographical region and the variability of macrolide resistance determinants carried by otherwise undistinguishable isolates strongly suggest that acquisition of macrolide resistance determinants is also ongoing.

The changes in the clonal composition of the population could have a profound impact on the prevalence of the macrolide resistance phenotypes as well as on overall resistance. To test whether a reduced diversity of the population could be implicated in the changes in macrolide resistance, the SID and 95% CIs for each study year in the period 1998-2006 were calculated (Figure 3.3.3). As expected, the initial overwhelming dominance of the T12-*emm*22-ST46 clone [30] resulted in the lowest SID being determined for the population expressing the MLS_B phenotype; this then increased steadily until 2000, in parallel with the decrease in the prevalence of this clone. The second decline

in the prevalence of the MLS_B phenotype, occurring between 2001 and 2003 (Figure 3.3.1), was associated with stable and higher SIDs, indicating that this decrease could not be attributed solely to changes in the prevalence of a single clone, as occurred in 1998, or to a lack of clonal diversity of the MLS_B isolates (Figure 3.3.3). The SID of the population presenting the M phenotype was significantly higher than that of the population presenting the MLS_B phenotype up to 2001, during a period of increased prevalence of the M phenotype, but the two populations remained undistinguishable for the remainder of the study period (SIDs with overlapping CIs).

This latter observation does not support a difference in mobility of the genetic elements carrying each of the macrolide resistance determinants, which would be expected to result in a higher diversity of the population associated with the more mobile genetic elements. It is noteworthy that the steady, although not significant, decline in the diversity of the populations displaying both macrolide resistance phenotypes from 2001 to 2003, occurring in a population increasingly dominated by a few clones, was followed by a period of greater variability that paralleled the changes in the prevalence of each phenotype. However, large changes in the diversity of the population, apparent in the fluctuations of SID, continued to occur in the somewhat stable situation concerning macrolide resistance and phenotypes reached in the period 2005–2006. This suggests that, although the clonal composition of the population and the prevalence of the macrolide resistance phenotypes are certainly related, the overall make-up of the population probably results from the dynamic interaction between these two factors, and neither can be assumed to be the cause of the changes observed in the other.

This study has several limitations that may have influenced the results presented. In spite of the location of the laboratories throughout Portugal, which covered a large fraction of the population, only close to 1200 isolates were recovered. Two major factors are behind the relatively small number of isolates: (i) the management of pharyngitis relies mainly on rapid antigen tests and clinical criteria, with culture being infrequently performed; and (ii) although the laboratories were asked to submit all GAS associated with the diagnosis of pharyngitis, an audit to ensure compliance, which may vary in this type of study, was not undertaken [26]. The increase in the number of isolates that was particularly apparent in the period 2005-2006 was found among all participating laboratories, and we attribute this mainly to increased compliance with our request to send all GAS isolated in

pharyngitis cases. The alternative explanation would be that there is an increased incidence of infections caused by macrolide susceptible GAS which, consequently, would bias the estimates of macrolide resistance. However, this seems unlikely, because outbreaks of macrolide-susceptible GAS would probably be restricted to a few regions and would not be a national phenomenon, and no changes were observed in the proportions of isolates from each of the major regions considered. More importantly, the proportion of isolates expressing the MLS_B phenotype remained approximately constant in spite of the increase in the total number of isolates, whereas only the proportion of isolates expressing the M phenotype decreased in recent years (Figure 3.3.1).

This study showed a decline in macrolide resistance among GAS causing pharyngitis in Portugal. These changes were accompanied by clonal variations, but the great diversity of the populations displaying each of the macrolide resistance phenotypes precludes the simple explanation that these could be solely attributed to strong fluctuations in a few resistant clones. The results underscore the importance of considering both the clonal structure of the bacterial population and antibiotic consumption when attempting to explain the prevalence of resistant isolates.

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CHAPTER 3.4

MACROLIDE RESISTANCE IN *STREPTOCOCCUS PYOGENES* IN PORTUGAL (2007-2011): DIMINISHING RESISTANCE AND CLONAL INSTABILITY, IN SPITE OF HIGH MACROLIDE CONSUMPTION

SUMMARY

Macrolide resistance among *Streptococcus pyogenes* (Group A streptococci – GAS) in Portugal decreased in 1999-2006, and this was accompanied by alterations in the prevalence of macrolide resistance phenotypes and clonal composition of the population. The aims of this study were to gain further insights into the changes in overall macrolide resistance, of the fluctuations of macrolide resistance phenotypes and of the clonal structure of the macrolide resistant population of GAS recovered in 2007-2011 in Portugal. We report continuing changes in macrolide resistance phenotypes and a persistent decline in overall erythromycin resistance, despite continued high macrolide use and increased consumption. Characterization by *emm* typing, T typing, pulsed-field gel electrophoresis (PFGE) profiling and multilocus sequence typing revealed a marked increase in T11-*emm*11-ST403 cMLS$_B$ isolates, the disappearance of the T3-*emm*3-ST315 M clone, and changes in the prevalence of previously identified GAS clones. Unexpectedly, the decline in erythromycin resistance and the decreasing prevalence of the MLS$_B$ phenotype were accompanied by a high consumption of long-acting and intermediate-acting macrolides that are known to select for resistance and the *erm*(B) gene. In spite of high macrolide consumption, the decline in macrolide resistance detected since 2000 continued to occur, in a population with high clonal instability, emphasizing the importance of considering factors other than antibiotic consumption in explaining the prevalence of resistance.

INTRODUCTION

Streptococcus pyogenes or Lancefield group A streptococcus (GAS) is an important human pathogen causing a broad spectrum of infections from acute pharyngitis to severe invasive disease. Macrolides and lincosamides constitute suitable alternatives to the treatment of streptococcal infections, especially in patients allergic to β-lactams.

Two mechanisms of resistance to macrolides have been described in *Streptococcus pyogenes*: target site modification and active efflux. Target site modification is caused by the presence of a methylase encoded by the *erm*(B) gene or *erm*(TR) gene [belonging to the *erm*(A) class], that results in resistance to most macrolides, lincosamides and streptogramin B (the MLS_B phenotype) [17]. The expression of the *erm* genes can be constitutive or inducible, generating the $cMLS_B$ phenotype and the $iMLS_B$ phenotype, respectively. Both classes of *erm* genes were found associated with transposons with the capacity to transfer the resistance traits to susceptible strains by conjugation [6]. The other mechanism, responsible for resistance to 14- and 15-membered ring macrolides (the M phenotype) but not to clindamycin, is mediated by a membrane associated pump encoded by the *mef* determinants. Although the majority of GAS presenting the M phenotype carried the *mef*(A) variant [13], *mef*(E) was also detected in *S. pyogenes* [10].

In some countries, like Spain, Greece, Italy, Slovenia and Portugal, high macrolide resistance rates in *S. pyogenes* have been reported [8, 19, 21, 24, 26] while lower resistance rates were reported in other European countries [26, 32]. Likewise, some differences were also found in the prevalence of macrolide resistance phenotypes and the clonal composition of macrolide resistant GAS populations.

Increases in macrolide resistance are often associated with high antimicrobial consumption and several studies have already shown a correlation between the decrease in macrolide resistance and the decrease in antibiotic consumption [2, 27, 32]. Moreover, some studies showed an association between specific macrolides (intermediate-acting and long-acting macrolides) in selecting for resistance and in the selection of a particular macrolide resistance phenotype [18]. However, the circulating clones and the fluctuations in the clonal composition of the population can also be responsible for alterations in the prevalence of macrolide resistance and of the macrolide resistance phenotypes [8, 29].

In Portugal, a steady decline in macrolide resistance in GAS was reported between 1999 and 2006, from 20% in 1999 to 12% in 2006. This was accompanied by fluctuations of the macrolide resistance phenotypes and of the clonal composition of the population [29]. However, these fluctuations did not simply reflect fluctuations of the overall GAS population. In a study comparing the macrolide resistant and susceptible GAS populations in Portugal, we found that particular *emm* types and PFGE clusters were associated with macrolide resistance while others were associated with macrolide susceptibility and the changes in macrolide resistance were accompanied by fluctuations in *emm* types and their associated lineages [28]. The differences found between the macrolide resistant and susceptible populations indicated that the macrolide resistant population has its own dynamics.

The aims of this study were to document any changes in macrolide resistance and in the characteristics of resistant isolates in 2007-2011. We report ongoing fluctuations of macrolide resistance phenotypes and a continuing decline in overall erythromycin resistance, despite continued high macrolide use and discuss their possible causes.

MATERIALS AND METHODS

Bacterial isolates and identification

A total of 2632 *S. pyogenes* recovered from throat swabs, associated with a diagnosis of tonsillo-pharyngitis were collected from 32 microbiology laboratories located throughout Portugal from January 2007 to December 2011. The laboratories were asked to submit all non-duplicate *S. pyogenes* isolated from outpatients during the study period. The isolates were distributed in the study period as follows: 519 in 2007, 491 in 2008, 551 in 2009, 558 in 2010 and 513 in 2011. Isolates were identified to the species level by colony morphology, β-hemolysis on sheep blood agar, and a commercial latex-agglutination technique (Slidex Strepto A, BioMérieux, Marcy l'Etoile, France). In this collection, 139 isolates (5.3%) were erythromycin-resistant, and only these isolates were characterized further.

Antimicrobial susceptibility testing and macrolide resistance phenotype

Susceptibility to erythromycin, clindamycin and tetracycline (Oxoid, Basingstoke, UK) was tested using disk diffusion according to CLSI recommendations [9]. The macrolide resistance phenotype was determined according to a double disk test previously described [20]. Bacitracin susceptibility was determined for all isolates by disk diffusion using disks containing 0.05 U of bacitracin (Oxoid, Basingstoke, UK) as previously described [30].

PCR determination of the macrolide and tetracycline resistance genotype

Total bacterial DNA was isolated according to the methodology described by the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). PCR reactions to determine which of the macrolide resistant determinants [*erm*(B), *erm*(A) or *mef*] was present, were performed as described previously [12]. To discriminate between *mef*(A) and *mef*(E) genes, the *mef* gene was amplified by PCR as previously described [29].

PCR reactions for the detection of tetracycline resistant determinants, *tet*(K), *tet*(L), *tet*(M) and *tet*(O) were performed for all isolates included in this study, as previously described [29]. *Streptococcus dysgalatiae* subs. *equisimilis* SH533, SH523 and 645040

carrying *tet*(M), *tet*(O) and *tet*(L) respectively and an *Escherichia coli* strain carrying the pST181 plasmid with the *tet*(K) gene were used as positive controls.

T and *emm* typing

T typing was performed by slide agglutination using sera for types 1, 2, 3, 4, 6, 8, 9, 11, 12, 13, 18, 22, 23, 25, 28, 5/27/44, 14/19 and Imp.19 (Hemolytic streptococcus Typing Antisera for Group A (T-typing) SEIKEN, Denka Seiken, Tokyo, Japan), according to the manufacturer's instructions. *emm* typing was performed as described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Amplification products were purified using the High Pure PCR Purification Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, sequenced using primer emmseq2 and the DNA sequences were searched against the *emm* sequences deposited in the CDC database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

PFGE was performed as previously described [30]. The MLS_B isolates were digested with SmaI, and the isoschizomer Cfr9I was used for the isolates with the M phenotype, which were not digested by SmaI [30]. Bionumerics software (Applied-Maths, Sint-Martens-Latem, Belgium) was used to create UPGMA (unweighted pairgroup method with arithmetic mean) dendrograms of the SmaI or Cfr9I fragment patterns. The Dice similarity coefficient was used, with optimisation and position tolerance settings of 1.0 and 1.5, respectively. PFGE clusters were defined as isolates with $\geq$ 80% similarity [7].

A PFGE-based cluster was considered to be a major lineage if it included more than five isolates. MLST analysis was performed in at least 60% of randomly selected isolates of each *emm* type that was represented by more than 3 isolates (n=96) as previously described [11] and allele and sequence type (ST) were attributed using the MLST database (spyogenes.mlst.net). The relationships between the MLST STs were determined using the goeBURST [14] algorithm implemented in PHYLOVIZ [15] with the complete *S. pyogenes* database available at spyogenes.mlst.net.

Statistical analysis

Trends in macrolide resistance were evaluated using the Cochran-Armitage test for trend [3]. The Simpson's index of diversity and corresponding 95% confidence intervals were used to evaluate the clonal diversity of the isolates for each year since 1998 [7].

RESULTS

Antimicrobial susceptibility testing and macrolide and tetracycline resistance phenotype

During the five years of the study, the overall rate of erythromycin resistance was 5.3%, a lower value than the one reported in 2004-2006 (13.2%). A decreasing trend in macrolide resistance was noted in the period 2007-2011(10% to 2%, Cochran-Armitage test for trend, $p<0.001$).

A total of 95 of the 139 macrolide resistant isolates (68.3%) presented the $cMLS_B$ phenotype, 43 (30.9%) the M phenotype and only one isolate (0.8%) presented the $iMLS_B$ phenotype. The decline in macrolide resistance in this period was due to the decline of isolates presenting both macrolide resistance phenotypes, ($p<0.001$ and $p=0.037$ for MLS_B and M isolates, respectively (Figure 3.4.1).

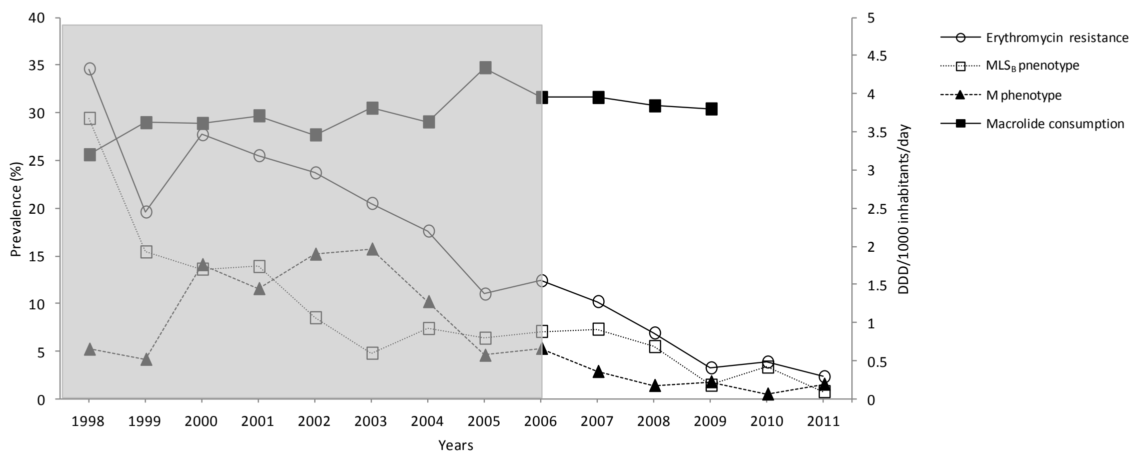

FIGURE 3.4.1 Erythromycin resistance and prevalence of macrolide resistance phenotypes in Portugal during the period 1998–2011. Open circles and solid lines represent the proportion of erythromycin-resistant group A streptococci among those causing pharyngitis. Broken lines represent the proportion of each phenotype in the population. Solid triangles represent the proportion of isolates of the M phenotype. Open squares represent the proportion of isolates of the MLS_B phenotype. Solid squares represent the macrolide consumption, measured by defined daily doses/1000 inhabitants/day [1]. The shaded area represents previously published data [29, 31]

Resistance to tetracycline was found in 59% (n=82) of the isolates. With the exception of two isolates expressing the M phenotype, all the tetracycline resistant isolates expressed the MLS_B phenotype. The distribution of the tetracycline resistant isolates

among the study years was as follows: 33 in 2007, 21 in 2008, 7 in 2009, 17 in 2010 and 4 in 2011. Resistance to bacitracin was found in 11 isolates, all expressing the $cMLS_B$ phenotype.

Identification of resistance determinants

All isolates tested carried a single macrolide resistant determinant. Among the $cMLS_B$ isolates, all but one carried the *erm*(B) gene. The exception was one isolate carrying the *erm*(A) determinant. This was also the resistant determinant carried by the single isolate presenting the $iMLS_B$ phenotype included in this collection. Almost all the M isolates carried the *mef*(A) gene, the exception was one isolate that yielded a PCR product indicating the presence of the *mef*(E) gene. This isolate was also also tetracycline resistant and carried the *tet*(M) determinant.

Among the tetracycline resistant isolates, *tet*(M) was found in 90.2% of the isolates (n=74), while 2 isolates (2.4%) carried the *tet*(O) gene. These two isolates also carried the *erm*(A) resistance determinant and belonged to the same *emm* type and PFGE cluster (see below). In six of the tetracycline resistant isolates, no resistant determinant was detected by PCR. None of the other *tet* determinants was found among the isolates studied.

Clonal characterization

The characteristics of 139 GAS isolates included in this study are summarized in Table 3.4.1. All 139 isolates were typable by PFGE using either SmaI or Cfr9I and nine major lineages were identified, containing 118 isolates (84.9%), while the remaining 21 isolates (15.1%) were included in minor PFGE clusters (containing four or fewer isolates) or had unique PFGE patterns. The PFGE patterns of representative isolates from each major cluster are shown in Figure 3.4.2 and the characteristics of the major lineages found in this study are shown in table 3.4.1.

MLST analysis was performed in 96 isolates (70%). We identified 3 novel alleles among the genes used in MLST, one in *gki* (*gki*116), and two in *gtr* (*gtr*91 and *gtr*92). The *gtr* allele 92 presented a 1-nt deletion that was predicted to result in a truncated form of the protein. New allele combinations producing novel STs were also noted and were assigned ST numbers 657 to 659. The new alleles and the novel STs were submitted to the *S. pyogenes* MLST database (spyogenes.mlst.net). Among the 96 isolates, 18 STs were

found. In all cases, isolates of the same *emm* type presented the same ST or STs of the same clonal complex (Table 3.4.1)

Table 3.4.1. Characteristics of the 139 macrolide resistant GAS in Portugal

emm type (no. of isolates)	PFGE cluster[a]	T types (no. of isolates)[b]	Macrolide resistance phenotype [genotype] (no. of isolates)	Tetracycline resistance phenotype [genotype] (no. of isolates)[c]	ST (no. of isolates)[d]
11(61)	A_{53}(53)	11(50), NT(2), 9(1)	cMLS$_B$ [*erm*(B)] (53)	R [*tet*(M)](49), [ND] (3); S(1)	[403(35), 562(1)]
	G_7(7)	11(7)	cMLS$_B$ [*erm*(B)] (7)	R [*tet*(M)](7)	403(5)
	Other (1)	11(1)	M [*mef*(A)](1)	R [ND](1)	ND
22(16)	C_{10}(9)	12(9)	cMLS$_B$ [*erm*(B)] (9)	R [*tet*(M)](8), [ND] (1)	46(7)
	Other (7)	12(7)	cMLS$_B$ [*erm*(B)] (9)	R [*tet*(M)](6), [ND] (1)	46(4)
12(15)	E_8(8)	12(5), NT(2), 9(1)	M [*mef*(A)](8)	S(8)	36(5)
	Other (7)	12(5), NT(2)	M [*mef*(A)](4)[*mef*(E)](1); cMLS$_B$ [*erm*(B)](2)	R [*tet*(M)](1); S(6)	36(5)
28(11)	B_{11}(11)	28(11)	cMLS$_B$ [*erm*(B)] (11)	S(11)	52(8)
4(8)	F_8(8)	4(8)	M [*mef*(A)](8)	S(8)	[39(5), 658(1)]
1(7)	H_7(7)	1(7)	M [*mef*(A)](7)	S(7)	[28(1), 618(1), 659(3)]
75(6)	I_6(6)	25(6)	M [*mef*(A)](6)	S(6)	657(4)
6(4)	D_8(4)	25(6)	M [*mef*(A)](3), cMLS$_B$ [*erm*(B)](1)	S(4)	[382(2), 411(1)]
9(4)	D_8(3)	9(2), NT(1)	M [*mef*(A)](3)	S(3)	75(2)
	Other (1)	9(1)	M [*mef*(A)](1)	S(1)	75(1)
Other[e]	Multiple[f]	Multiple[g]	cMLS$_B$ [*erm*(B)] (4) [*erm*(TR)](1); iMLS$_B$ [*erm*(TR)](1); M [*mef*(A)](1)	R [*tet*(M)] (3), [*tet*(O)] (2); S(2)	Multiple[h]

[a] Each major lineage is designated by a capital letter, followed by a number in subscript that indicates the number of isolates grouped in that lineage; whenever a PFGE cluster included less than 5 isolates of that particular *emm* type, it was not discriminated and it was designated by "other".
[b] NT, non-typeable
[c] ND (Not determined): Did not yield any amplification product for the genes tested
[d] 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] 6 *emm* types (7 isolates): 77(2), 3(1), 44(1), 73(1), 78(1) and 87(1)
[f] 5 PFGE clusters
[g] 6 T types: 13(2), 11(1), 28(1), 5/27/44(1), 9(1), NT(1)
[h] 4 STs: 63(2), 406(1), 409(1), 62(1)

The prevalence of the major *emm* types was not stable during the study period, and this instability was noted in the previously studied periods (1998-2006) (Figure 3.4.3). To evaluate if the diversity of the *emm* types varied during the period 1998-2011, the Simpson's Index of diversity and the corresponding 95% confidence intervals were calculated for each year. The results are summarized in Figure 3.4.4.

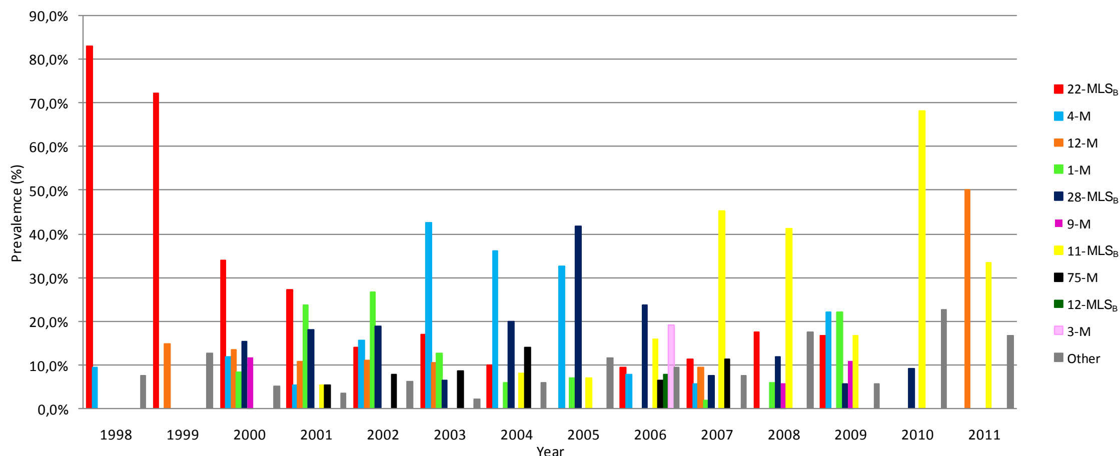

FIGURE 3.4.3. Proportion of each of the major lineages (defined by macrolide resistance phenotype and *emm* type) that accounted for >5% of macrolide-resistant *Streptococcus pyogenes* isolated in Portugal between 1998 and 2011. Whenever an *emm* type was expressed by fewer than 5% of the isolates recovered in that year, it was not discriminated and it was designated by "other".

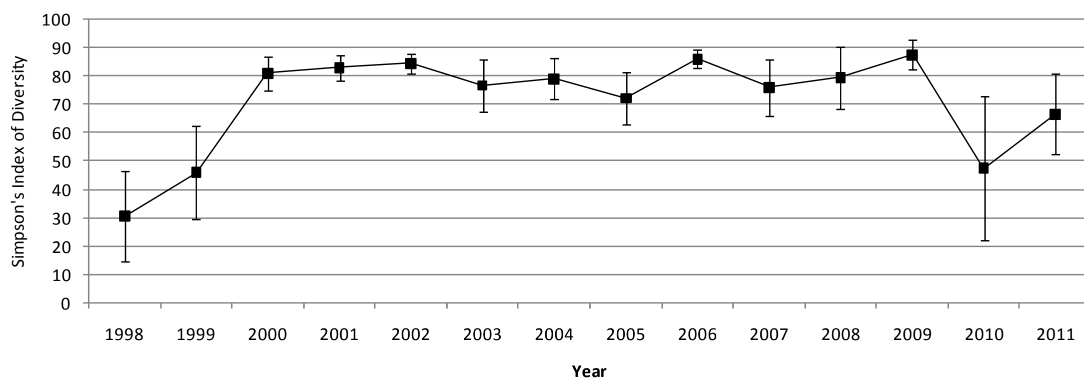

FIGURE 3.4.4. Simpson's index of diversity for the population of macrolide-resistant group A Streptococcus (1998–2011). Simpson's index of diversity and corresponding 95% CIs were calculated for the *emm* types of macrolide-resistant *Streptococcus pyogenes* isolates from each study year.

DISCUSSION

From 2007 to 2011, a decrease in erythromycin resistance rate among *S. pyogenes* responsible for pharyngitis was reported. The decreasing trend in macrolide resistance already detected in the period 1999-2006 [29], continued in this study period (2007-2011), reaching the lowest value of macrolide resistance in GAS recorded in Portugal (2% in 2011).

In Portugal, tetracycline resistance among macrolide resistant GAS is usually associated with the $cMSL_B$ phenotype and in pharyngitis isolates, the tetracycline resistant determinant found was always *tet*(M) [30]. In this study, we documented for the first time in Portugal, the presence of the *mef*(E) gene in a GAS isolate, that also carried *tet*(M). The *tet*(O) was found in two MLS_B isolates (one $iMLS_B$ and one $cMLS_B$ isolate), which also carried *erm*(A) and presented T13 and *emm*77. Because macrolide and tetracycline resistance determinants are carried in the same genetic elements, it was suggested that co-selection could explain changes in macrolide resistance among *S. pyogenes* [22] and we have already demonstrated that between 1998 and 2003 the decrease in tetracycline accompanied the decrease in the proportion of $cMLS_B$ isolates [31]. Among all the *S. pyogenes* isolates recovered from tonsillo-pharingytis patients in Portugal, a significant decrease in tetracycline resistance from 8% in 2007 to 2% in 2011 was noted ($p<10^{-6}$, Cochran Armitage test for trend), which could be attributed to a 54% decrease in ambulatory tetracycline consumption from 2000 to 2009 [25]. However, macrolide consumption in 2000, measured in defined daily doses (DDD)/1000 inhabitants/day, was twice as high as tetracycline consumption and in 2009 it was fivefold higher [25]. The high rates of tetracycline resistance among the macrolide resistant isolates in the last years of the study could simply be reflecting the high proportion of T11-*emm*11-ST403 isolates, carrying *erm*(B) and *tet*(M) determinants.

Ecological studies have associated changes in macrolide consumption with the prevalence of resistant isolates [16, 27, 32]. A randomized, double-blind, placebo-controlled study showed that azithromycin (a long-acting macrolide) was more efficient than clarithromycin (an intermediate-acting macrolide) in the selection of macrolide resistant streptococci of the oral commensal flora, although only the latter selected for the *erm*(B) gene [18]. The importance of this finding resides in the fact that the same genetic

elements conferring macrolide resistance are widely spread among streptococci [6, 13] and that commensal streptococci are able to transfer these elements to pathogenic species constituting a reservoir of resistance genes [23].

The situation documented in Portugal is in contrast with these findings. If we consider the entire period from 1998 to 2011, we are now able to show that the increase in macrolide consumption in the country was accompanied by a decrease in overall macrolide resistance in GAS. The macrolide consumption increased 35% from 3.20 DDD/1000 inhabitants/day in 1998 to a peak of 4.34 in 2005 [1]. From then on, consumption remained high and approximately constant until 2009 [1]. Moreover, the consumption of intermediate-acting and long-acting macrolides increased steadily during this period such that in 2009 Portugal was the third biggest consumer of long acting macrolides in Europe with 1.47 DDD/1000 inhabitants/day, which represents a 50% increase relatively to the values of 1998 (0.98 DDD/1000 inhabitants/day) [1]. The consumption of intermediate-acting macrolides was also high in this period, peaking in 2005 at 2.60 DDD/1000 inhabitants/day [1]. Moreover, the increase in consumption of intermediate-acting macrolides, shown to select for the *erm*(B) gene, coincided with a decrease in the proportion of isolates presenting the MLS_B phenotype and an increase in the M phenotype observed in the period 1999-2003, and with the decrease of both phenotypes from 2004 onwards (Figure 3.4.1). It could be argued perhaps that there was insufficient selective pressure to maintain a high macrolide resistance rate in GAS, but consumption in Portugal has remained well above the value of 2 DDD/1000 inhabitants/day, believed to trigger an increase in macrolide resistant GAS [16]. Although we have no data concerning macrolide consumption since 2009, it seems unlikely that a rapid decline in macrolide use, especially of long- and intermediate-acting macrolides would explain the variations seen in 2009-2011, because any changes in macrolide use are expected to have a delayed effect on resistance rate [16]. Moreover, mathematical models predict that once resistance was selected [5], only drastic reductions in antibiotic use can be hoped to lead to reduced resistance rates, and this was not the case, since overall macrolide consumption remained high, and at levels of use above the threshold for selection of resistance, at least until 2009.

Changes in the clonal composition, already described in the period 1998 to 2006 [29, 30] continued in the period 2007 to 2011. The most notable change is the increase of

T11-*emm*11-ST403 isolates, which accounted for 10% of the isolates in the period of 2004-2006, and represent 44% of the GAS isolates in the period 2007 to 2011. A high prevalence of the T11-*emm*11-ST403 lineage was also reported in Spain, but its spread was associated with an increase in macrolide resistance [4], contrary to our findings in Portugal. Other differences include the disappearance of the T3-*emm*3-ST315 lineage, which was associated with macrolide susceptibility in Portugal [28] and rarely described among macrolide resistant GAS in other countries. All the genetic lineages identified among macrolide resistant GAS appear to be disseminated in other European countries [4, 19].

In Belgium, a decrease in macrolide resistance rate was noted after a reduction of macrolide consumption and in a situation of low levels of resistance, the proportion of isolates of a single clone (*erm*(A)-*emm*77 isolates), with a low fitness cost, increased significantly [32]. The analysis of the prevalence of the major *emm* types circulating in Portugal (Figure 3.4.3) showed that between 1998 and 2011, each *emm* type seems to be dominant for periods of 3-4 years, without a clear decrease in the proportion of a single clone accompanying the decrease in macrolide resistance. To test if the decrease in macrolide resistance was accompanied by a decrease in the diversity of the population we calculated the SID of the *emm* types and 95% confidence intervals for each study year between 1998 and 2011 (Figure 3.4.4). As already reported [31], the dominance of the *emm*22 in the two first years of the study resulted in lower SIDs; between 2000 and 2009, the SIDs were higher but not stable with no significant yearly variation (showing overlapping confidence intervals). Finally, in 2010 and 2011, the population showed a lower diversity again, with a great predominance of *emm*11 isolates. However, this decrease in the diversity of the population cannot be invoked to explain the decrease in macrolide resistance because in these two years the number of resistant isolates was very low and, more importantly, because the decrease in macrolide resistance was noted even when the SIDs were stable (2000-2009).

Macrolide consumption still stands as the main driver of macrolide resistance in streptococci and this is well documented [16, 27, 32]. However, the association between clonal fluctuations in the population and macrolide resistance phenotypes must have a role in the dynamics of macrolide resistant GAS population, as already recognized [8, 28]. Here we showed a decline of macrolide resistance among GAS associated with pharyngitis

to a level as comparable to the lowest in Europe (including countries with low consumption) [1, 26, 32] . This decrease occurred in spite of a high macrolide use, especially of intermediate-acting and long-acting macrolides previously implicated in selection of resistance. Particularly, the increase in the M phenotype observed in some years and the decrease in the MLS_B phenotype observed since 2001 was unexpectedly accompanied by an increase in the consumption of long-acting macrolides, which have a high potential for selection of the *erm*(B) gene.

These changes were accompanied by variations in the clonal composition of the population, which are part of the natural dynamics of the macrolide resistant GAS, independently of the behavior of the general GAS population. All these results underscore the importance of considering both the clonal structure of the bacterial population as well as antibiotic consumption when attempting to explain the prevalence of resistant isolates.

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CHAPTER 4

DIFFERENCES BETWEEN MACROLIDE-RESISTANT AND -SUSCEPTIBLE *STREPTOCOCCUS PYOGENES*: IMPORTANCE OF CLONAL PROPERTIES IN ADDITION TO ANTIBIOTIC CONSUMPTION

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SUMMARY

A steady decline in macrolide resistance among *Streptococcus pyogenes* (group A streptococci [GAS]) in Portugal was reported during 1999 to 2006. This was accompanied by alterations in the prevalence of macrolide resistance phenotypes and in the clonal composition of the population. In order to test whether changes in the macrolide-resistant population reflected the same changing patterns of the overall population, we characterized both macrolide-susceptible and -resistant GAS associated with a diagnosis of tonsillo-pharyngitis recovered in the period from 2000 to 2005 in Portugal. Pulsed-field gel electrophoresis (PFGE) profiling was the best predictor of *emm* type and the only typing method that could discriminate clones associated with macrolide resistance and susceptibility within each *emm* type. Six PFGE clusters were significantly associated with macrolide susceptibility: T3-*emm*3-ST406, T4-*emm*4-ST39, T1-*emm*1-ST28, T6-*emm*6-ST382, B3264-*emm*89-ST101/ST408, and T2-*emm*2-ST55. Four PFGE clusters were associated with macrolide resistance: T4-*emm*4-ST39, T28-*emm*28-ST52, T12-*emm*22-ST46, and T1-*emm*1- ST28. We found no evidence for frequent ongoing horizontal transfer of macrolide resistance determinants. The diversity of the macrolide-resistant population was lower than that of susceptible isolates. The differences found between the two populations suggest that the macrolide-resistant population of GAS has its own dynamics, independent of the behavior of the susceptible population.

INTRODUCTION

Streptococcus pyogenes (Lancefield group A streptococci [GAS]), is the most common bacterial agent implicated in acute pharyngitis and can also cause a variety of skin and soft tissue infections, as well as severe invasive disease. Although penicillin remains the antibiotic of choice in the treatment of GAS infections, macrolides and lincosamides are recommended as suitable alternatives for patients who are allergic to penicillin [4].

High macrolide resistance in GAS was reported in most southern European countries, such as Spain, Greece, Italy, and Portugal [14, 15, 19, 22, 25, 27], but this was not a characteristic of all European countries [22]. Although differences in the prevalence of particular macrolide resistance genotypes and *emm* types were documented, the majority of these *emm* types shared the same resistance determinants, suggesting a broad geographical dissemination of a few clones. Isolates fully susceptible to macrolides that share the same pulsed-field gel electrophoresis (PFGE) profiles or the same sequence types (STs) as these major clones have been described, and several resistance determinants have been associated with each resistant lineage. Taken together, these data imply that independent acquisition of resistance genes by the same prevalent clones followed by local dissemination could have also played a role in conditioning the successful macrolide resistance phenotypes and clones in particular geographic locations.

An association between certain *emm* types and macrolide resistance was documented [1, 7, 16, 17, 23, 28], but in most of these studies the characterization of the *S. pyogenes* isolates was limited to *emm* typing. However, it was recently shown that this typing technique is not sufficient to unambiguously identify GAS clones. For the precise identification of genetic lineages, *emm* typing should be complemented with other typing methods, such as PFGE or multilocus sequence typing (MLST) [5]. In Portugal, we noted a steady decline in macrolide resistance among *S. pyogenes* isolates in 1999 to 2006, decreasing from 20% in 1999 to 12% in 2006 [25]. This was accompanied by large fluctuations of the macrolide resistance phenotypes, as well as changes in the clonal composition of the population [25–27]. Given these results, we hypothesized that the changing patterns of macrolide resistance could also be reflecting fluctuations in the overall population, implying that important causes for this fluctuation lay outside antibiotic usage and reflected other selective forces acting on the entire GAS population.

The aims of this study were to characterize the macrolide-susceptible population of GAS and to compare it with the macrolide resistant population to determine how much the dynamics of macrolide- resistant isolates could be mirroring the behavior of the overall GAS population.

MATERIALS AND METHODS

Bacterial isolates and identification

A total of 1,606 *S. pyogenes* isolates recovered from throat swabs and associated with a diagnosis of tonsillo-pharyngitis were collected from 32 microbiology laboratories located throughout Portugal from January 2000 to December 2005. In this period, erythromycin resistance declined from 28% in 2000 to 11% in 2005 [25]. Pharyngitis is frequently managed in Portugal without a microbiological investigation, with these being performed mostly for epidemiological purposes. On the other hand, the availability of rapid antigen tests means that an isolate will not always be recovered, even when an etiological diagnosis is sought. The participating laboratories were asked to submit all nonduplicate *S. pyogenes* isolates obtained from outpatients during the study period, but no audit was performed to ensure compliance, and this is known to be variable in this kind of study. The combination of these factors may have contributed to a lower number of isolates than anticipated, but we have no reason to suspect that there was a bias in the isolates that were submitted. The isolates were distributed in the study period as follows: 214 in 2000, 216 in 2001, 270 in 2002, 230 in 2003, 284 in 2004, and 392 in 2005. Isolates were identified to the species level by colony morphology, beta-hemolysis on sheep blood agar, and a commercial latex agglutination technique (Slidex Strepto A; bioMérieux, Marcy l'Etoile, France). A total of 803 isolates, randomly chosen and representing 50% of the total collection, were characterized. Among this group were 155 macrolide resistant isolates (19.3%). The distribution of the macrolide-resistant isolates included in this study was as follows: 23 in 2000, 19 in 2001, 23 in 2002, 38 in 2003, 30 in 2004, and 22 in 2005. These isolates represent a subset of the total number of macrolide-resistant isolates found during 2000 to 2005 (n=318).

Antimicrobial susceptibility testing

Susceptibilities to erythromycin and clindamycin were tested by disk diffusion according to the recommendations of CLSI [6] and were reported previously [25–27]. The macrolide resistance phenotype was determined according to previously published procedures [27].

DNA extraction

Total bacterial DNA was isolated according to the methodology described by the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm).

T and *emm* typing

emm typing was performed as described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Amplification products were purified using the High Pure PCR purification kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and sequenced using primer emmseq2 [2], and the DNA sequences were searched against the *emm* sequences deposited in the CDC database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

Pulsed-field gel electrophoresis (PFGE) and Multilocus sequence typing MLST

Pulsed-field gel electrophoresis (PFGE) was performed as previously described [26]. All the macrolide-susceptible isolates were digested with SmaI, and isoschizomer Cfr9I was used only for isolates presenting the M phenotype [26]. The BioNumerics software (Applied-Maths, Sint-Martens-Latem, Belgium) was used to create unweighted-pair group method with arithmetic mean (UPGMA) dendrograms of the SmaI or Cfr9I fragment patterns. The Dice similarity coefficient was used, with optimization and position tolerance settings of 1.0 and 1.5, respectively. PFGE clusters were defined as groups of isolates with $\geq$ 80% similarity in the dendrogram [5]. A PFGE-based cluster was considered to be a major lineage if it included $\geq$ 10 isolates. Clusters that included between 5 and 10 isolates were considered minor PFGE clusters. Major lineages were identified by uppercase letters and minor clones by lowercase letters. The subscript number in the PFGE cluster designation identifies the number of isolates included in the cluster.

Multilocus sequence typing (MLST) analysis was performed for at least 30% of randomly selected isolates of each *emm* type that was represented by more than 10 isolates (n=282) as previously described [8], and allele and sequence type (ST) were attributed using the *S. pyogenes* MLST database (spyogenes.mlst.net). The relationships between the MLST STs were determined using the goeBURST algorithm [9] implemented in PHYLOViZ [10] with the complete *S. pyogenes* database available at spyogenes.mlst.net.

Statistical analysis

In order to compare the probability of a given *emm* type or PFGE clone being more associated with macrolide resistance or susceptibility, the odds ratios (OR) and the corresponding p values, corrected for multiple testing through the false-discovery rate (FDR) linear procedure [3], were calculated by reference to all other *emm* types and PFGE clones.

Adjusted Wallace (AW) coefficients were used to compare partitions, and Simpson's index of diversity (SID) and the corresponding 95% confidence intervals (CIs) were used to evaluate the clonal diversity of the macrolide-susceptible and -resistant isolates [5, 24]. Trends were evaluated using the Cochran-Armitage test of trend, and yearly variability was tested using the Fisher exact test. p values of <0.05 were considered significant.

RESULTS

Clonal characterization

The characteristics of the 803 isolates included in this study, as well as their distribution during the study period, are summarized in Table 4.1. Among the 34 different *emm* types found, the most frequent were *emm*4 (15.2%, n=122), *emm*1 and *emm*3 (9.6%, n=77 each), *emm*12 (9.3%, n=75), *emm*6 (7.2%, n=58), and *emm*89 (7%, n=56). The remaining 28 *emm* types accounted for 42.1% of the isolates (n=338). Among the 16 T types found in the collection, the most prevalent were T4 (13.8%, n=111), T12 (12.5%, (n=100), T1 (9.7%, n=78), T28 (8.5%, n=68), and B3264 (8.3%, n=67). Slightly under 8% of the isolates (n=64) were T nontypeable.

The 803 isolates were classified into 62 PFGE clusters, including 19 major lineages representing 684 isolates (85%) (Figure 4.1). Nine minor PFGE clusters were found, grouping 60 isolates (7.5%). In most cases, each PFGE cluster had a dominant macrolide resistance phenotype (susceptible, MLS_B, or M), *emm* type, T type, and STs belonging to the same clonal complex (Table 1). The MLS_B isolates were grouped mainly into PFGE cluster H_{38} (48%, n=30) and cluster K_{27} (33%, n=21), while 49% of isolates presenting the M phenotype (n=45) were included into cluster G_{49}. MLST analysis was performed for 282 isolates (35.1%). We identified 4 novel alleles among the genes used in MLST, two in *gki* (*gki*108 and *gki*109), one in *mutS* (*muts*63) and one in *recP* (*recp*101). New allele combinations producing novel STs were also noted and were numbered ST559 to ST569 and ST634 to ST639. The new alleles and the novel STs were submitted to the *S. pyogenes* MLST database (spyogenes.mlst.net). Isolates of the same *emm* type presented mostly the same ST or STs of the same clonal complex (Table 4.1).

TABLE 4.1– Properties of the 803 GAS isolates collected from tonsillo-pharyngitis in Portugal

emm (no. isolates)	PFGE clusters (R/S)[a]	T type [b] (no. isolates)	ST(no. isolates)[c]	2000	2001	2002	2003	2004	2005
4 (122)	B_{66} (1/64)	4(63), 3(1), NT(1)	[39(12), 566(1), 635(1)]	2	2	10	12	10	29
	G_{49} (45/2)	4(39), B3264(2), 13(1), 2/4(1), 5/27/44(1), 6(1), 12(1), 1(1)	[39(16), 561(1), 637(1), 638(1)]	4	0	6	16	13	8
	Other (2/8)	4(8), 6(1), 2(1)	39(6), 15(1), 55(1)	0	0	1	2	0	7
1(77)	C_{62}(0/62)	1(61), NT(1)	[28(16), 567(1)]	7	6	8	7	20	14
	R_{12} (12/0)	1(10), 13(1), NT(1)	28(7)	1	2	4	4	0	1
	Other (1/2)	1(3)	28(1)	0	1	2	0	0	0
3(77)	A_{76} (0/76)	3(40), NT(18), 3/13(17), 5/27/44(1)	[406(14), 315(5), 15(4), 560(1)]	1	8	30	4	3	30
	Other (0/1)	NT(1)	15(1)	0	0	0	0	0	1
12(75)	D_{59} (8/42)	12(40), NT(9), 2(1)	[36(14), 551(1)]	9	10	6	11	5	9
	S_{10} (5/5)	12(9), NT(1)	36(5)	1	2	1	2	3	1
	A_9 (0/8)	12(6), NT(2)	36(3)	1	0	2	2	1	2
	Other (4/3)	12(6), 25(1)	36(3), 150(1)	3	0	4	0	0	0
6(58)	E_{58} (1/56)	6(51), NT(4), 5/27/44(1), 2(1)	[382(18), 411(1)]	13	10	8	4	2	20
	Other (0/1)	6(1)	382(1)	1	0	0	0	0	0
89(56)	F_{53} (1/51)	B3264(50), 3/13(1), 28(1)	[101(5), 408(5), 553(1), 568(3)]	1	8	5	11	15	12
	Other (0/4)	B3264(3), 5/27/44(1)	[101(2), 568(1)] 555(1)	0	1	0	0	1	2
75(49)	L_{25} (8/16)	25(24)	[150(7), 563(1)]	3	6	8	6	1	0
	Q_{13} (0/13)	25(13)	[150(3), 564(1), 639(1)]	1	1	2	2	5	2
	Other (4/8)	25(11), NT(1)	150(5), 481(1)	1	1	0	3	5	2
28(45)	H_{38} (26/7)	28(32), NT(1)	52(9)	4	8	5	3	5	8
	P_{13} (1/9)	28(10)	52(5)	0	2	3	1	3	1
	Other (0/2)	28(1), NT(1)	[52(1), 456(1)]	0	0	2	0	0	0

emm (no. of isolates)	PFGE clones (R/S)[a]	T type [b]	ST(n)[c]	2000	2001	2002	2003	2004	2005
2(38)	I_{32} (0/30)	2(29), 4(1)	[55(7), 634(1)]	4	6	3	3	9	5
	Other (2/6)	2(7), 12(1)	[55(4), 636(1)]	1	1	1	1	1	3
22(35)	K_{27} (21/4)	12(25)	[46(6), 389(1)]	7	7	1	4	5	1
	Other (6/4)	12(10)	[46(2), 389(1)], 52(2)	0	2	1	5	2	0
44/61(33)	J_{32} (1/30)	5/27/44(28), NT(2), 12(1)	[25(10), 554(1)]	0	0	0	2	14	15
	Other (0/2)	5/27/44(2)	555(1)	0	0	1	0	0	1
87(25)	N_{20} (0/18)	28(16), NT(2)	62(6)	4	3	2	3	3	3
	i_5 (0/5)	28(3), 1(1), NT(1)	62(2)	0	0	0	1	0	4
	Other (0/2)	28(1), 6(1)		0	0	0	0	0	2
9(23)	M_{24} (0/19)	9(19)	75(4)	8	3	3	1	2	2
	Other (3/1)	9(4)	75(4)	2	0	1	0	0	1
77(17)	b_8 (0/8)	13(8)	63(3)	2	1	1	2	2	0
	Other (0/9)	13(5), NT(2), 28(1), 2(1)	63(3)	1	2	5	0	0	1
78(15)	O_{15} (0/15)	11(15)	[253(5), 409(1)]	5	2	1	0	2	5
94(11)	D_{59} (0/9)	B3264(7), 1(1), NT(1)	89(4)	2	1	1	1	1	3
	Other (0/2)	B3264(2)		0	0	2	0	0	0
Other [d]	Multiple [e]	Multiple [f]	Multiple [g]	18	12	5	2	9	1

[a]PFGE clones defined as major lineages are designated by uppercase letters, and minor PFGE clones (10> n≥ 5) are designated by lowercase letters. Whenever a PFGE clone included fewer than 5 isolates of that particular *emm* type, it was not discriminated, and all such isolates were grouped under "other." The numbers of resistant (R) and susceptible (S) isolates are indicated in parenthesis.
[b]NT, nontypeable.
[c]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.
[d]18 *emm* types (47 isolates): 11 (9), 102 (9), 58 (5), 48 (3), 74 (2), 18 (2), 29 (2), 68 (2), 53 (2), st7700 (2), 118 (2), 43 (1), 73 (1), 70 (1), 64 (1), 25 (1), 5 (1), and st38 (1).
[e]Twenty-one PFGE clones.
[f]12 T types: NT (15), 11 (8), 13 (5), 3/13 (5), B3264 (3), 28 (3), 9 (2), 3 (2), 25 (1), 12 (1), 18 (1), and 1 (1).
[g]17 STs: 60 (3), 403 (2), 562 (2), 410 (2), 247 (1), 331 (1), 161 (1), 176 (1), 402 (1), 552 (1), 559 (1), 167 (1), 565 (1), 569 (1), 120 (1), 63 (1), and 12 (1)

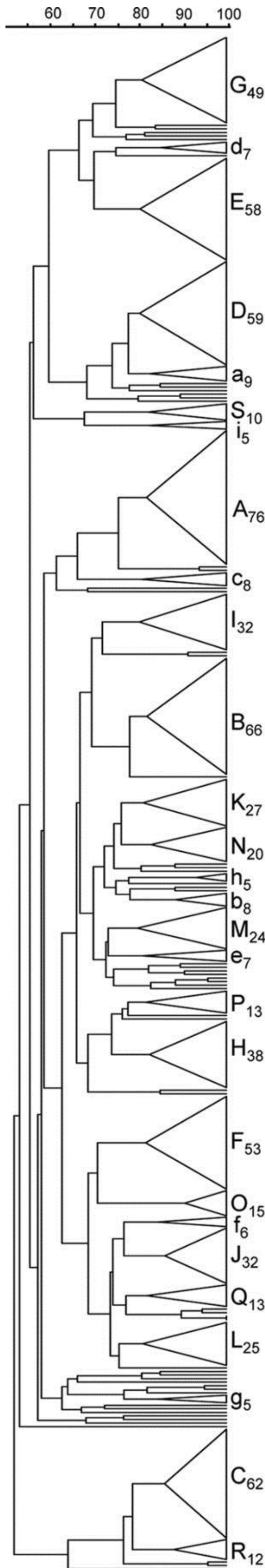

FIGURE 4.1 Dendrogram showing the PFGE profiles of the 803 GAS isolates. The dendrogram was constructed using the unweighted-pair group with arithmetic mean (UPGMA) method. Dice coefficients (percentages) are indicated in the scale above the dendrogram. For each PFGE cluster, a triangle proportional to the number of isolates is shown in the dendrogram. The clusters are designated by letters, with major clusters ($n \geq 10$) identified by uppercase letters and minor PFGE clusters ($10 > n \geq 5$) identified by lowercase letters. The subscript numbers indicate the number of isolates included in the cluster.

Differences between PFGE clusters and *emm* types between macrolide-resistant and -susceptible isolates

The SID values of all typing methods and the corresponding 95% CIs were determined for the macrolide-resistant and -susceptible subsets to ascertain whether there were differences in the diversities of the two populations (Table 4.2). For all the typing methods used to characterize the entire collection, the diversity of the macrolide-resistant population was lower than the diversity of the susceptible population.

This not only is evident from the lower number of different types found among macrolide-resistant isolates but also is reflected in the significantly lower SID values (Table 4.2). For the subset of isolates analyzed by MLST, the diversity of the macrolide- resistant population was also lower.

TABLE 4.2 - Simpson's index of diversity and 95% Confidence intervals ($CI_{95\%}$) of the typing methods used in the analysis of the 155 macrolide resistant isolates and 648 macrolide susceptible GAS isolates

Typing method	**SID ($CI_{95\%}$) [no. of partitions]**	
	Macrolide resistant isolates (n=155)	**Macrolide susceptible isolates (n=648)**
PFGE	0.851 (0.817-0.884) [22]	0.938 (0.932-0.944) [58]
emm typing	0.823 (0.792-0.854) [12]	0.927 (0.922-0.933) [34]
T typing	0.819 (0.787-0.850) [14]	0.924 (0.920-0.928) [16]
MLST	0.889 (0.853-0.924) [17]	0.959 (0.951-0.966) [52]

Among the *emm* types that were represented by more than 10 isolates, none included solely resistant isolates. However, some were found only in macrolide-susceptible isolates: *emm*3, *emm*87, *emm*77, *emm*78, and *emm*94. The calculation of OR supported the association of *emm*3 and *emm*87 with macrolide susceptibility ($p< 0.001$ and $p= 0.007$, respectively, both of which are significant for FDR) but not that of *emm*77, *emm*78, and *emm*94. *emm* types 6 and 89 were also associated with macrolide susceptibility, although both included one resistant isolate (both with a p value of <0.001, which is significant for FDR). On the other hand, *emm* types 4, 22, and 28 were associated with macrolide resistance (all with a p value of <0.001, which is significant for FDR).

Nine PFGE clusters that included only susceptible isolates were also identified (Table 4.1). In contrast, only one PFGE cluster, R_{12} (mostly T1-*emm*1-ST28), grouped

exclusively resistant isolates. All the other PFGE clusters included both susceptible and resistant isolates.

Similarly to what was found for *emm* types, the calculation of OR for the PFGE major clusters supported only the association of some clusters with the macrolide resistance phenotype. The association with susceptibility of PFGE clusters A_{76} (mostly T3-*emm*3-ST406), C_{62} (mostly T1-*emm*1-ST28), and I_{32} (mostly T2-*emm*2-ST55) was statistically supported (p<0.001, p<0.001, and p=0.002, respectively, all of which are significant for FDR). Additionally, PFGE clusters B_{66} (mostly T4-*emm*4-ST39), E_{58} (mostly T6- *emm*6-ST382), and F_{53} (mostly B3264-*emm*89-ST101/ST408), all including one resistant isolate, were also found to be associated with susceptibility (all with a p value of <0.001, which is significant for FDR). In contrast, PFGE clusters G_{49} (mostly T4-*emm*4-ST39), H_{38} (mostly T28 -*emm*28-ST52), K_{27} (mostly T12-*emm*22- ST46), and R_{12} (mostly T1-*emm*1-ST28) were associated with macrolide resistance (all with a p value of <0.001, which is significant for FDR).

Correspondence between typing methods and differences between macrolide-susceptible and -resistant isolates

The AW coefficient calculated for the relationship between PFGE and macrolide susceptibility was high ($AW_{PFGE \rightarrow Ery}$=0.715; 95% CI, 0.642 to 0.788), while it was extremely low for *emm* type ($AW_{emm \rightarrow Ery}$=0.095; 95% CI, 0.016 to 0.175). The AW coefficients also indicated that among the typing methods used in this work, PFGE was the best predictor of *emm* type. This was valid considering all the isolates included in the collection and the macrolide-resistant and -susceptible subsets ($AW_{PFGE \rightarrow emm,\ all}$=0.914; 95% CI, 0.889 to 0.939). The inverse relationship was significantly weaker ($AW_{emm \rightarrow PFGE,\ all}$ = 0.598; 95% CI, 0.565 to 0.630). When considering only the macrolide-resistant or -susceptible subsets, the relationship was much stronger ($AW_{emm \rightarrow PFGE,\ Ery^r}$=0.754 [95% CI, 0.658 to 0.850]; $AW_{emm \rightarrow PFGE,\ Ery^s}$ =0.787 [95% CI, 0.747 to 0.827]), reflecting the discrimination by PFGE of macrolide-resistant and macrolide-susceptible lineages of the same *emm* types. For the subset of isolates analyzed by MLST (n=282), the ST was an excellent predictor of *emm* type ($AW_{ST \rightarrow emm}$=0.963; 95% CI, 0.937 to 0.988), but the inverse relationship was significantly weaker ($AW_{emm \rightarrow ST}$ =0.678; 95% CI, 0.602 to 0.753).

Changes of macrolide resistance within *emm* types with time

Since particular genetic lineages were found to be associated with macrolide resistance, we tested whether the distribution of macrolide-resistant isolates changed with time within each of the *emm* types with ≥ 10 isolates in the studied period. Changes were noted in *emm*1 ($p = 0.016$) and *emm*4 ($p < 0.001$), but only the latter was significant for FDR. This indicates that while the proportion of macrolide-resistant isolates remained stable in time for most *emm* types, that for *emm*4 changed significantly. An increase in *emm*4 isolates was noted during the study period (Cochran-Armitage test for trend, $p<0.001$). However, this increase reflects the underlying dynamics of its two major PFGE clusters. The increase in recent years of the macrolide-susceptible PFGE cluster B_{66}, which peaked in the last year of the study, is in contrast to the dramatic increase of the macrolide-resistant PFGE cluster G_{49} in the early years, to a peak in 2003, and its current decline (Table 4.1).

DISCUSSION

The changes in the prevalence of macrolide resistance phenotypes noted in Portugal were accompanied by alterations in the clonal composition of the population [25]. At this point, it remained unclear whether all these changes were a reflection of large fluctuations in the overall GAS population or whether macrolide-resistant *S. pyogenes* isolates have their own dynamics. In this work, we compared macrolide-resistant and -susceptible GAS isolates in the period from 2000 to 2005 in order to gain new insights into the reasons behind the fluctuations reported for the resistant population.

In this study, a statistically significant association was found between macrolide resistance and *emm*4, *emm*22, and *emm*28, of which *emm*4 and *emm*22 were also found in association with macrolide resistance elsewhere [1, 16, 23, 29]. However, our findings are in contrast to a report from Korea that indicated a relationship between these *emm* types and macrolide susceptibility [12]. It was also possible to establish significant associations between macrolide susceptibility and *emm*3, *emm*6, *emm*87, and *emm*89. While *emm*3 was also previously reported in association with macrolide susceptibility in Spain [1] and in Italy [29], *emm*89 was previously found in association with macrolide resistance [29], contrary to our findings. Associations between certain *emm* types and macrolide resistance and susceptibility were reported in other studies but were not detected in Portugal, such as for *emm*12 and *emm*2, which were often associated with macrolide resistance [7, 17, 29], and *emm*1 isolates, which were frequently found in association with macrolide susceptibility [1, 16].

PFGE was the only typing method capable of differentiating resistant isolates of the same *emm* type. For *emm*4 and *emm*1, PFGE grouped the isolates into two major PFGE clusters (B_{66} and G_{49} for *emm*4 isolates and C_{62} and R_{12} for *emm*1 isolates), associated with macrolide susceptibility and resistance, respectively. Associations between some PFGE clusters and macrolide susceptibility that could not be detected by *emm* type alone were identified. This was the case for clusters C_{62} and R_{12}, including *emm*1 macrolide-susceptible and -resistant isolates, respectively, and for cluster I_{32}, including *emm*2 isolates. None of these *emm* types was statistically associated with macrolide susceptibility. These observations reinforce the usefulness and the importance of using PFGE to characterize GAS isolates.

In order to compare the two populations, the SID values for all the typing methods used in this study were calculated (Table 4.2). There were differences in the number of types and SID values between the macrolide-resistant and -susceptible subsets for PFGE, *emm* typing, and T typing. The diversity of the resistant population was lower than that of the susceptible subset by all three methods. The SID values for the fraction of the isolates characterized by MLST were also lower in the macrolide-resistant population.

The association of certain *emm* types and PFGE clones with macrolide resistance or susceptibility and the differences in the diversity of the two groups indicate that the macrolide-resistant isolates represent lineages distinct from the susceptible population. The discrepancies in associations between *emm* types and macrolide resistance found in different geographical regions could be due to the circulation of different lineages expressing these *emm* types in different areas.

If the diversity of the resistant population previously reported [25–27] was a reflection of the diversity of the overall GAS population, the *emm* types and the PFGE clusters would not show these associations, and the diversities of the two populations would be approximately the same. Some *emm* types are never or rarely found in resistant isolates, even though their prevalence in susceptible isolates is high. This is the case for *emm*3, *emm*6, and *emm*89. In 2002 and 2005, 22% and 15% of the isolates, respectively, were *emm*3, and no macrolide-resistant isolates were detected. Fifty seven out of the 58 *emm*6 isolates were macrolide susceptible, and while in 2005, 10% of the isolates presented this *emm* type, no acquisition of macrolide resistance determinants was detected. The same situation occurred with *emm*89, which increased throughout the study period, but a single resistant isolate was found. Taken together, these data suggest that there is limited ongoing transfer of macrolide resistance determinants.

The narrow group of resistant lineages is quite stable and has disseminated widely [11, 13, 18, 20, 21, 28]. Whether their success is dependent solely on their association with macrolide resistance or may also reflect other clonal properties remains to be shown. The characterization of contemporaneous macrolide- resistant and -susceptible isolates revealed distinct populations that changed independently in the study period.

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CHAPTER 5

SCARLET FEVER IS CAUSED BY A LIMITED NUMBER OF *STREPTOCOCCUS PYOGENES* LINEAGES AND IS ASSOCIATED WITH THE EXOTOXIN GENES *SSA*, *SPEA* AND *SPEC*

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SUMMARY

Background: Several outbreaks of scarlet fever caused by *Streptococcus pyogenes* were recently reported. Historically considered a toxin-mediated disease, dependent on the production of the exotoxins SpeA and SpeC, a strict association between scarlet fever and these exotoxins is not always detected. The aims of this study were to characterize the scarlet fever isolates recovered in a Lisbon hospital and to identify any distinctive characteristics of such isolates.

Methods: We characterized a collection of 303 pharyngeal *S. pyogenes* collected between 2002 and 2008. 101 were isolated from scarlet fever patients and 202 were associated to a diagnosis of tonsillo-pharyngitis. Isolates were characterized by T and *emm* typing, pulsed field gel electrophoresis (PFGE) profiling and superantigen gene profiling.

Results: The diversity of the scarlet fever isolates was lower than the pharyngitis isolates. Specific lineages of *emm*87, *emm*4 and *emm*3 were overrepresented in scarlet fever isolates but only one PFGE major lineage was significantly associated with scarlet fever. Multivariate analysis indicated associations of *ssa*, *speA* and *speC* with scarlet fever.

Conclusions: In non-outbreak conditions, scarlet fever is caused by a number of distinct genetic lineages. The lower diversity of these isolates and the association with specific exotoxin genes indicates that some lineages are more prone to cause this presentation than others even in non-outbreak conditions.

INTRODUCTION

Streptococcus pyogenes or Lancefield group A streptococcus (GAS) is an important human pathogen responsible for a wide range of infections including relatively mild infections such as tonsillo-pharyngitis, scarlet fever and impetigo, as well as severe infections such as streptococcal toxic shock syndrome and necrotizing fasciitis [11]. Scarlet fever is usually associated with streptococcal throat infection, but it may also occur due to infections at other sites and is characterized by skin rash and a “strawberry tongue” [11]. In the 19th and early 20th centuries, it was seen as a significant cause of childhood morbidity and mortality [21] with a significant cyclical behavior [13]. Although currently considered a relatively rare and mild disease, a number of scarlet fever outbreaks were recently reported [5, 14, 18, 22, 27]. In Shangai, 114 cases of scarlet fever in children were reported over a 1-month period in June 2011 [5], while in Hong Kong more than 900 cases of scarlet fever were recorded between January and July 2011[19]. These recent outbreaks motivated an increased interest in this syndrome and in the possible bacterial factors involved.

S. pyogenes produces many virulence factors and among them are the secreted pyrogenic exotoxins (Spe), which act as superantigens. Some of these superantigens are also believed to be responsible for some of the clinical signs of scarlet fever. Early studies indicated that the skin rash characteristic of scarlet fever is caused by the production of SpeA, but in its absence, SpeC could also cause identical symptoms [30]. However, other studies did not detect an association of these exotoxins with scarlet fever [5, 12, 30, 31]. In Canada, the combined presence of SpeA and SpeC was more frequently found in scarlet fever isolates than in pharyngitis isolates [28]. A correlation between scarlet fever and SpeA, but not SpeC was reported in the USA [30, 31]. The recent outbreak reported in China failed to associate SpeA with scarlet fever, although a significant association between SpeC, SpeJ, SSA and SMEZ and scarlet fever was detected [5, 27].

It was suggested that the acquisition of mobile elements, carrying specific superantigen combinations, could be related to the occurrence of scarlet fever outbreaks [27].

The sequencing of the hypervariable region of the *emm* gene (*emm* typing) [2] which encodes the M protein, a major virulence factor produced by *S. pyogenes*, has been

the most widely used tool for epidemiological studies of GAS. However, it was recently shown that this typing technique is not sufficient to unambiguously identify GAS clones and should be complemented with other typing methods, such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST), for a better characterization of GAS clones [4].

While some scarlet fever outbreaks were reported as being caused by isolates of a particular *emm* type [5, 14], others were found to be multiclonal outbreaks, with scarlet fever isolates of the same *emm* type occurring across multiple genetic lineages [27]. In other studies, temporal changes in the prevalence of the *emm* types associated with scarlet fever were reported [6–8, 29]. In some cases, this was related to the natural fluctuations of *emm* types [8], while in others a relationship with decreases in antimicrobial resistance and changes of the clonal composition of the population were detected [6].

The aims of this study were to characterize a collection of *S. pyogenes* isolates responsible for scarlet fever and to compare it with non scarlet fever pharyngeal isolates, in order to gain insights into the possible bacterial characteristics associated with this syndrome. With this in mind, we undertook the phenotypic and molecular characterization of 101 scarlet fever isolates and 202 non-scarlet fever isolates. All the isolates were characterized by antimicrobial susceptibility testing, T and *emm* typing , PFGE and a recently developed method to screen GAS isolates for the presence of 11 SAg genes, that allowed the detection of all the known allelic variants of these genes [16]. No *emm* type or SAg profile were associated with the ability to cause this disease. In contrast, one PFGE cluster and specific superantigen combinations were associated with scarlet fever.

MATERIALS AND METHODS

Bacterial isolates and identification

A total of 101 *S. pyogenes* isolates recovered from throat swabs and associated with a diagnosis of scarlet fever were collected in the microbiology laboratory of a large teaching hospital in Lisbon, between January 2002 and December 2008. For each scarlet fever isolate, two non-scarlet fever isolates (isolates recovered from pharyngeal exudates, associated with tonsillo-pharyngitis which will be referred as pharyngitis isolates) were randomly chosen from among the isolates recovered in that year in the same laboratory. A single isolate from each patient was included in the study. The total number of isolates analyzed was n=303 and the median age of patients with scarlet fever was 5 yrs and of those with pharyngitis was 4 yrs. Isolates were identified to the species level by colony morphology, β-hemolysis on sheep blood agar, and a commercial latex-agglutination technique (Slidex Strepto A, BioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing and macrolide resistance phenotype

Susceptibility to penicillin, erythromycin and clindamycin (Oxoid, Basingstoke, UK) was tested using disk diffusion according to CLSI recommendations [9]. The macrolide resistance phenotype was determined according to a double disk test previously described [20]. Bacitracin susceptibility was determined for all isolates by disk diffusion using disks containing 0.05 U of bacitracin (Oxoid, Basingstoke, UK) as previously described [26].

PCR determination of the macrolide resistance genotype

Total bacterial DNA was isolated according to the methodology described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). PCR reactions to determine which of the macrolide resistance determinants (*erm*(B), *erm*(A) or *mef*) was present and to discriminate between *mef*(A) and *mef*(E) were performed as previously described [15, 25].

T and *emm* typing

T typing was performed by slide agglutination using sera for types 1, 2, 3, 4, 6, 8, 9, 11, 12, 13, 18, 22, 23, 25, 28, 5/27/44, 14/19 and Imp.19 (Hemolytic streptococcus Typing Antisera for Group A (T-typing) SEIKEN, Denka Seiken, Tokyo, Japan), according to the manufacturer's instructions and previously published procedures [17].

emm typing was performed as described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Amplification products were purified using the High Pure PCR Purification Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, sequenced using primer emmseq2[1] and the DNA sequences were searched against the *emm* sequences deposited in the CDC database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

SAg genes profiling

The presence of 11 SAg genes was tested in two multiplex PCR, as previously described, using *speB* and *speF* as internal controls [16]. One PCR reaction was used to detect the presence of *speF*, *speG*, *speH*, *speJ*, *speK*, *ssa* and *smeZ* and the genes *speA*, *speB*, *speC*, *speI*, *speL* and *speM* were screened in another reaction [16].

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as previously described [26]. Bionumerics software (Applied-Maths, Sint-Martens-Latem, Belgium) was used to create UPGMA (unweighted pairgroup method with arithmetic mean) dendrograms of the SmaI or Cfr9I fragment patterns. The Dice similarity coefficient was used, with optimisation and position tolerance settings of 1.0 and 1.5, respectively. PFGE clusters were defined as isolates with ≥ 80% similarity [4]. A PFGE-based cluster was considered to be a major lineage if it included more than five isolates.

Statistical analysis

In order to compare the probability of a given *emm* type, PFGE clone or SAg profiles to be associated with scarlet fever, the odds ratios (OR) and the corresponding p-values, corrected for multiple testing through the False Discovery Rate (FDR) linear procedure [3] were calculated by reference to all other *emm* types, PFGE clones and SAg

profiles, as appropriate. Multivariate logistic regression was used in exploratory analysis to detect associations with scarlet fever, using each of the SAg genes as predictor variables. After considering all SAg genes, only SAg genes with $p<0.05$ were retained in the final set of variables that were tested for interaction terms. Since the *spe*L and *spe*M genes always co-occurred in our collection, they were considered as a single variable in our analyses.
The R (version 2.12.0) software and the Epiools package (http://medepi.com/epitools/) were used for the statistical analysis.

The Simpson's index of diversity (SID) and the corresponding 95% confidence intervals were used to evaluate diversity [4].

RESULTS

Antimicrobial susceptibility testing and macrolide resistance phenotype

All isolates included in this study were susceptible to penicillin. Macrolide resistance was detected in 35 isolates (12%). The majority of macrolide resistant isolates presented the M phenotype (n=19); 15 isolates presented the $cMLS_B$ phenotype and only one the $iMLS_B$ phenotype. All the M isolates carried the *mef*(A) gene and the *erm*(B) resistant determinant was present in all the MLS_B isolates. One $cMLS_B$ isolate carried both the *mef*(A) and *erm*(B) genes. All macrolide resistance isolates included in the scarlet fever group (n=10) presented the M phenotype. The MLS_B isolates were all included in the pharyngitis subset.

Bacitracin resistance was detected in nine isolates, all *emm*28. The majority (n=7) also presented the $cMLS_B$ phenotype. No bacitracin resistance was detected among the scarlet fever group.

Clonal characterization

The characteristics of the 303 isolates included in this study are summarized in Table 5.1. A total of 22 *emm* types and 17 T types were found. Approximately 8% of the isolates were T non-typeable (Table 5.1).

All 303 isolates were typable by PFGE using either SmaI or Cfr9I and 33 PFGE clusters and 16 major lineages were identified, containing 272 isolates (89%). In most cases, each PFGE cluster was associated with a particular T type, *emm* type, and SAg profile (Table 5.1).

TABLE 5.1 – Properties of the 303 GAS isolates collected from tonsillo-pharyngitis in Portugal

emm type (no. of isolates)	PFGE cluster (SF/NSF)[a]	T types[b] (no. of isolates)	SAg profiles (no. of isolates)
4 (58)	A_{49}(22/26)	4(46), 3(1), NT(1)	23(44), 22(2), 47(1), 91(1)
	I_{10}(6/4)	4(8), 1(1), 2/4(1)	23(5), 30(3), 27(2)
1(37)	B_{33}(8/24)	1(32)	10 (23), 3(9)
	Other (5)	1(5)	10(3), 3(1), 8(1)
3(36)	C_{30}(13/13)	3(17), 3/13(4), NT(5)	8(20), 88(3), 89(1), 92(1), 37(1)
	L_8(6/2)	3(6), 3/13(1), 5/27/44(1)	8(5), 88(2), 52(1)
	Other (0/2)	NT(2)	8(1), 33(1)
87(26)	D_{28}(15/11)	28(20), 2(1), NT(5)	20(21), 44(1), 21(1), 27(1), 18(1), 12(1)
12(24)	E_{20}(3/14)	12(13), NT(4)	16(6), 33(4), 17(2), 73(1), 35(1), 77(1), 85(1), 34(1)
	Other (0/7)	12(7)	16(2), 35(2), 17(1), 76(1), 14(1)
28(18)	J_{10}(1/7)	28(8)	27(7), 21(1)
	M7(1/6)	28(6), NT(1)	24(5), 27(2)
	Other (0/3)	28(3)	24(2), 3(1)
89(17)	G_{17}(2/15)	B3264(17)	29(12), 27(3), 43(1), 46(1)
44(15)	F_{18}(4/7)	5/27/44(7), 12(2), 25(1), NT(1)	67(5), 32(5), 76(1)
	Other (3/1)	5/27/44(3), NT(1)	67(1), 8(1), 16(1), 87(1)
6(14)	H_{14}(3/9)	6(12)	2(8), 26(2), 43(1), 46(1)
	Other (1/1)	6(2)	2(2)
75(14)	F_{18}(1/5)	25(6)	42(3), 25(1), 45(1), 83(1)
	Other (0/8)	25(8)	39(4), 25(1), 81(1), 82(1), 84(1)
2(8)	K_9(0/4)	2(4)	31(3), 79(1)
	Other (2/2)	2(3), 4(1)	31(2), 78(1), 20(1)
11(7)	O_7(1/6)	11(6), 12(1)	5(4), 16(1), 17(1), 86(1)
9(7)	N_7(1/6)	9(6), 3(1)	40(5), 74(1), 38(1)
78(6)	P_5(1/3)	11(2), 28(1), B3264(1)	29(3), 80(1)
	Other (0/2)	11(1), 28(1)	29(2)
22(5)	K_9(0/2)	12(2)	19(1), 21(1)
	Other(1/2)	11(1), 12(1), 5/27/44(1)	16(1), 38(1), 75(1)
Other[c]	Other[d]	Multiple[e]	Multiple[f]

[a] PFGE clusters defined as major lineages are designated by uppercase letters, followed by the number of isolates included in that PFGE cluster, in subscript. 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". The number of scarlet fever (SF) and non-scarlet fever (NSF) isolates are indicated in parenthesis.
[b] NT, nontypeable
[c] 7 *emm* types (n=11): 77 (n=3), 48(n=2), 94(n=2), 71(n=1), 58(n=1), 18(n=1), 102(n=1)
[d] Seven PFGE clones (n=3 SF/ n=8 NSF)
[e] Six T types: NT(n=4), B3263(n=2), 13(n=2), 28(n=1), 3(n=1), 8(n=1)
[f] Eight SAg profiles: 27(n=3), 45(n=2), 46(n=1), 30(n=1), 29(n=1), 17(n=1), 60(n=1), 4(n=1)

SAg profiling revealed that all 303 isolates carried the *spe*B and *spe*F genes. As expected, the chromosomally located exotoxins were much more frequent than those carried in bacteriophages (Table 5.2).

TABLE 5.2. Odds ratios (OR), 95% Confidence intervals ($CI_{95\%}$) and corresponding p- values of associations between SAg genes and scarlet fever.

Gene	Scarlet fever (no. of isolates)	Pharyngitis (no. of isolates)	Univariate analysis		Multivariate analysis	
			OR (95% CI)	p	OR (95% CI)	p
speH	12	34	0.667 [0.299-1.401]	0.310	0.995 [0.323-2.955]	0.992
ssa	73	75	4.392 [2.548-7.726]	**<0.001**[b]	6.041 [3.084-12.458]	**<0.001**[d]
speI	10	23	0.856 [0.348-1.966]	0.845	2.542 [0.824-8.026]	0.105
speK	26	43	1.281 [0.7-2.316]	0.387	0.961 [0.402-2.198]	0.926
speJ	39	75	1.065 [0.63-1.79]	0.803	1.777 [0.907-3.567]	0.098
smeZ	100	196	3.052 [0.363-142.11]	0.431	0.578 [0.052-13.877]	0.675
speG	72	174	0.401 [0.213-0.752]	**0.003**[c]	0.47 [0.181-1.189]	0.114
speL/M[a]	4	21	0.356 [0.086-1.098]	0.075	0.886 [0.177-3.519]	0.870
speA	37	54	1.582 [0.917-2.719]	0.085	4.418 [1.936-10.652]	**0.001**[d]
speC	65	127	1.066 [0.631-1.815]	0.899	2.125 [1.036-4.511]	**0.044**[d]

[a] *speL* and *speM* were always detected together
[b] Significant association with scarlet fever in univariate analysis
[c] Significant association with pharyngitis in univariate analysis
[d] Significant association with scarlet fever in multivariate analysis

SAg gene profiling analysis revealed the presence of 60 different profiles, of which 29 were unique profiles and 20 were new profiles relative to our previous SAg survey [16] (see Table, Supplemental Digital Content 1, which details all the superantigen gene profiles identified in our collection).

Differences between *emm* types, exotoxins and PFGE clusters between scarlet fever and pharyngitis isolates

With the exception of T typing, in all the other typing methods used to characterize the isolates, the diversity of the scarlet fever group was lower than the diversity of the pharyngitis isolates (Table 5.3).

TABLE 5.3 - Simpson's index of diversity and 95% Confidence intervals ($CI_{95\%}$) of the typing methods used in the analysis of the 101scarlet fever isolates and 202 pharyngitis GAS isolates.

Typing method	**SID ($CI_{95\%}$) [no. of partitions]**	
	Scarlet fever isolates (n=101)	**Pharyngitis isolates (n=202)**
PFGE	0.880 (0.849-0.911) [19]	0.938 (0.926-0.949) [32]
emm typing	0.853 (0.816-0.890) [18]	0.921 (0.909-0.933) [20]
SAg profile	0.900 (0.865-0.935) [33]	0.951 (0.940-0.962) [48]
T typing	0.870 (0.835-0.904) [15]	0.910 (0.898-0.922) [15]

In the scarlet fever group, *emm*4, *emm*3, *emm*87 and *emm*1 were the most prevalent and together accounted for 72% of the isolates. In the pharyngitis group *emm*4 was also the most frequently found, followed by *emm*1, *emm*12, *emm*3 and *emm*28 with 54% of the isolates included in these *emm* types. The calculation of OR identified *emm*87, *emm*4 and *emm*3 (p=0.009 for *emm*87 and *emm*4 and p=0.013 for *emm*3) as associated with scarlet fever, although FDR correction did not support any of these associations.

Among the 16 major PFGE clusters, only one (K_9) contained solely pharyngitis isolates and no major lineage included exclusively scarlet fever isolates. Four PFGE clusters were associated with scarlet fever including clusters A_{49}, grouping *emm*4 isolates, C_{30} and L_8, both including *emm*3 isolates (p=0.007 for C_{30}, p=0.032 for A_{49} and p=0.018 for L_8). However, PFGE clone D_{28}, grouping all *emm*87 isolates and two isolates representing *emm*2 and *emm*18, was the only PFGE cluster whose association was robust to FDR correction (p=0.002).

The analysis of the SAg profiles revealed that in the scarlet fever isolates, 33 different profiles were found and 10 included 2 or more isolates. Among the pharyngitis isolates, 48 different profiles were detected. There were differences in the prevalence of SAg profiles between the two collections, with the calculation of OR identifying profiles

20, 23 and 32 as associated with scarlet fever (p=0.004 for SAg profile 20, p=0.013 for SAg profile 23 and and p=0.044 for SAg profile 32), but none of these associations was supported by FDR. In a univariate analysis, *ssa* was associated with cases of scarlet fever, while *speG* was underrepresented in this group. In contrast, in a multivariate analysis, not only *ssa* but also *speA* and *speC* were significantly associated with scarlet fever (Table 5.2). The logistic regression model using the presence of these three genes as explanatory variables presented a good fit to the data (Hosmer-Lemeshow goodness of fit test, p=0.263). The inclusion of interaction terms between these three variables was not supported since the interaction terms were not significant and therefore no interaction terms were considered.

DISCUSSION

The macrolide resistance rate detected among scarlet fever isolates was similar to the overall macrolide resistance reported in Portugal [24], indicating that macrolide resistant clones were not particularly excluded from this population. Interestingly, the MLS_B phenotype was never detected in the scarlet fever group of isolates but the numbers were too low to draw any conclusions.

The SID values for all typing methods used in this study showed that the diversity of scarlet fever isolates was lower than the diversity of the pharyngitis isolates (Table 5.3). This supports the hypothesis that particular genetic lineages are more prone to cause this syndrome than others.

For most cases, isolates of the same *emm* type were divided into more than one PFGE cluster, although this distinction was not supported by SAg profling. In general, isolates with the same *emm* type, grouped in different PFGE clusters, share the same SAg profile. The exceptions to this were *emm*28 isolates, where the prevalent SAg profile was different in the two lineages representing these isolates (Table 5.1). These results are in agreement with other studies, that reported an association of *emm* with exotoxin profile [10, 16].

The calculation of OR and the correction for multiple testing through FDR only supported the association of the major lineage K_9, grouping mostly *emm*87 isolates, with scarlet fever. However, before the correction for multiple testing, other associations with scarlet fever were significant, such as the association of *emm*87, *emm*4 and *emm*3 and exotoxin profiles 20, 23 and 32. Although *emm*4 is among the most prevalent *emm* types described in scarlet fever [6–8, 29], the association of *emm*3 and *emm*87 with scarlet fever was rarely described. The recent scarlet fever outbreaks were caused by *emm*12 isolates [5, 27] and a food-borne outbreak of scarlet fever in adults, in China, was linked to *emm*75 [12]. In a day care center in Mexico, *emm*22, macrolide resistant *S. pyogenes* were responsible for a scarlet fever outbreak [14]. In our collection, these *emm* types are rare among scarlet fever isolates (Table 5.1). In some epidemiological studies, the *emm* types more frequently associated with scarlet fever were also frequently found in the general GAS population [5, 23]. In agreement with this, a study conducted in Portugal found

*emm*3, *emm*87 and *emm*4 among the most frequent *emm* types in pharyngitis, with *emm*3 and *emm*87 associated with macrolide susceptibility [24].

The outbreaks reported in Hong Kong and Shangai were caused by *emm*12 isolates carrying *speC*, *speJ*, *ssa* and *smeZ* and in one of the outbreaks *speA* was conspicuously absent from outbreak isolates [5, 27]. *speC* and *ssa* were found to be in the same prophage, designated by ΦHKU.vir, different from the usual prophage carrying these two genes (Φ9429.1). In the scarlet fever group of isolates analyzed here, three isolates presented *emm*12 and only one carried both exotoxins simultaneously, indicating that the lineage found in Hong Kong and Shangai was largely absent from Portugal. Isolates carrying simultaneously *speC* and *ssa* were frequent (n=86) and were overrepresented in isolates recovered from cases of scarlet fever ($p<0.001$), suggesting that bacteriophages related to the ones found in these recent outbreaks could be circulating in Portugal. In agreement with these observations, a multivariate analysis identified *speC* and *ssa*, together with the historically recognized *speA* gene, as being associated with scarlet fever. These toxins are present, individually or together, in the majority of the isolates representing the *emm* types and major PFGE lineages that were found associated with scarlet fever.

Our study has the limitation of having been conducted in a single hospital. This could have made it prone to biases due to the circulation of a limited number of lineages and the occurrence of outbreaks, in spite of all patients included being outpatients. However, we do not believe this to be the case, since the diversity of the isolates causing pharyngitis is similar to the one found in a nationwide study (Table 5.3) [24], indicating that the GAS clones isolated in this hospital reflect the clones found in the whole country. We have also not noted any significant temporal clustering of cases, beyond those seasonally expected (data not shown). We characterized a relatively small number of isolates but scarlet fever in Portugal, similarly to pharyngitis, is mostly managed without a microbial investigation, with this being performed mostly for epidemiological purposes. On the other hand, the availability of rapid antigen tests means that an isolate will not always be recovered, even when an ethiological diagnosis is sought. However, we do not know of any bias when seeking culture and the isolates in our collection are expected to reflect the characteristics of the overall GAS population causing scarlet fever.

Our study indicates that in a non-outbreak context, a large number of distinct genetic lineages have the ability to cause scarlet fever cases, although the diversity of these

isolates is lower than those causing pharyngitis, indicating that the first are not a simple reflection of the latter. We have confirmed that the presence of the historically recognized exotoxin encoding gene *speA* is associated with isolates recovered from scarlet fever. We have also shown that the genes *speC* and *ssa*, whose importance was documented in the context of recent outbreaks of scarlet fever, were also associated with scarlet fever in non-outbreak conditions in Portugal. Although scarlet fever undoubtedly results from an interplay between the infecting bacteria and the host, the presence of these bacterial genes can be used to monitor the strains with potential to cause the cyclical resurgences of this disease that are known to occur.

Supplemental Digital Content 1 – Superantigen genes (SAg) profiles identified in 303 GAS isolates

SAg profile (no. of isolates)	*speA*	*speC*	*speG*	*speH*	*speI*	*speJ*	*speK*	*speM*	*speL*	*ssa*	*smeZ*	*emm* type (no. of isolates)
2 (10)	+	+	+	-	-	-	+	-	-	-	+	6(10)
3 (11)	+	+	+	-	-	+	-	-	-	-	+	1(10), 28(1)
4 (1)	+	+	+	-	-	-	-	+	+	-	+	18(1)
5 (4)	+	+	+	-	-	-	-	-	-	-	+	11(4)
8 (28)	+	-	+	-	-	-	+	-	-	+	+	3(26), 1(1), 44(1)
10 (26)	+	-	+	-	-	+	-	-	-	-	+	1(26)
12 (1)	-	+	+	+	+	+	-	-	-	+	+	87(1)
14 (1)	-	+	+	+	-	-	-	-	-	+	+	12(1)
16 (11)	-	+	+	+	+	-	-	-	-	-	+	12(8), 1(1), 22(1), 44(1)
17 (5)	-	+	+	+	-	-	-	-	-	-	+	12(3), 58(1), 11(1)
18 (1)	-	+	+	-	-	+	+	-	-	+	+	87(1)
19 (1)	-	+	+	-	-	-	+	-	-	+	+	22(1)
20 (22)	-	+	+	-	-	+	-	-	-	+	+	87(21), 2(1)
21 (3)	-	+	+	-	-	-	-	-	-	+	+	87(1), 22(1), 28(1)
22 (2)	-	+	-	-	-	-	-	+	+	+	+	4(2)
23 (49)	-	+	-	-	-	-	-	-	-	+	+	4(49)
24 (7)	-	+	+	-	-	+	+	-	-	-	+	28(7)
25 (2)	-	+	+	-	-	-	+	+	+	-	+	75(2)
26 (2)	-	+	+	-	-	-	+	-	-	-	+	6(2)
27 (18)	-	+	+	-	-	+	-	-	-	-	+	28(9), 89(3), 4(2), 87(1),
29 (18)	-	+	+	-	-	-	-	-	-	-	+	89(12), 78(5), 48(1)
30 (4)	-	+	-	-	-	-	-	-	-	-	+	4(3), 77(1)
31 (5)	-	+	+	-	-	-	-	+	+	-	-	2(5)
32 (5)	-	-	+	+	+	+	-	-	-	+	+	44(5)
33 (5)	-	-	+	+	+	-	-	-	-	-	+	12(4), 3(1)
34 (1)	-	-	+	+	-	+	-	-	-	-	+	12(1)
35 (3)	-	-	+	+	-	-	-	-	-	-	+	12(3)
37 (1)	-	-	+	-	-	-	+	-	-	+	+	3(1)
38 (2)	-	-	+	-	-	+	-	-	-	+	+	22(1), 9(1)
39 (4)	-	-	+	-	-	-	-	+	+	+	+	75(4)
40 (5)	-	-	+	-	-	-	-	-	-	+	+	9(5)
42 (3)	-	-	+	-	-	-	+	+	+	-	+	75(3)
43 (2)	-	-	+	-	-	-	+	-	-	-	+	6(1), 89(1)
44 (1)	-	-	+	-	-	+	-	-	-	-	+	87(1)
45 (3)	-	-	+	-	-	-	-	+	+	-	+	94(2), 75(1)
46 (2)	-	-	+	-	-	-	-	-	-	-	+	77(1), 89(1)
47 (1)	-	-	-	-	-	-	-	-	-	-	+	4(1)
52 (1)	+	+	+	-	-	-	+	-	-	+	+	3(1)
60 (1)	+	-	+	+	+	+	-	-	-	-	+	71(1)
67 (6)	-	-	+	+	-	+	-	-	-	+	+	44(6)

SAg profile (no. of isolates)	*speA*	*speC*	*speG*	*speH*	*speI*	*speJ*	*speK*	*speM*	*speL*	*ssa*	*smeZ*	*emm* type (no. of isolates)
73 (1)	-	-	+	-	+	-	-	-	-	-	+	12(1)
74 (1)	-	-	+	-	-	+	+	-	-	+	+	9(1)
75 (1)	-	-	-	-	+	+	+	-	-	+	+	22(1)
76 (2)	-	-	+	+	-	-	-	-	-	+	+	12(1), 44(1)
77 (1)	-	-	+	+	+	-	-	-	-	+	+	12(1)
78 (1)	-	+	+	-	-	-	+	+	+	-	-	2(1)
79 (1)	-	+	+	-	+	-	-	+	+	-	-	2(1)
80 (1)	-	+	+	-	+	-	-	-	-	-	+	78(1)
81 (1)	-	+	+	-	+	-	-	+	+	-	+	75(1)
82 (1)	-	+	+	-	+	-	+	+	+	-	+	75(1)
83 (1)	-	+	+	-	+	+	+	+	+	-	+	75(1)
84 (1)	-	+	+	-	+	-	-	-	-	+	+	75(1)
85 (1)	-	+	+	-	+	+	-	-	-	+	+	12(1)
86 (1)	-	+	+	+	-	+	-	-	-	-	+	11(1)
87 (1)	-	+	+	+	-	+	-	-	-	+	+	44(1)
88 (5)	+	-	+	-	-	+	+	-	-	+	+	3(5)
89 (1)	+	-	+	+	-	-	-	-	-	+	+	3(1)
90 (1)	+	+	+	-	-	+	+	-	-	-	+	6(1)
91 (1)	+	+	+	-	-	-	-	-	-	+	+	4(1)
92 (1)	+	+	+	+	-	-	-	-	-	+	+	3(1)

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CHAPTER 6

GENERAL DISCUSSION

Streptococcus pyogenes is a pathogen with the ability to cause a wide variety of infections in its sole host, the human, ranging from uncomplicated infections in the upper respiratory tract or the skin, such as tonsillo-pharyngitis or impetigo, to severe, life threatening infections like necrotizing fasciitis or streptococcal shock toxic syndrome [12]. In spite of extensive use, penicillin remains the antibiotic of choice for the treatment of infections caused by GAS, but in cases of allergy, macrolides are a suitable alternative, with the additional advantage of having an oral route of administration. This is particularly important in children and in Portugal where there is no oral penicillin available. However, soon after its introduction in the clinical practice, macrolide resistance arose in clinical isolates of *S. pyogenes*. There are large geographical and temporal differences in the macrolide resistance rates and phenotypes, and the clonal composition of the population can also vary greatly. More importantly, the relationship between the antimicrobial consumption and macrolide resistance is not always so obvious, implying a role for the natural fluctuations of resistant clones in the prevalence of macrolide resistance rate and phenotypes.

Given this, the main goal of this Thesis was the characterization of *S. pyogenes* associated with tonsillo-pharyngitis, with a special emphasis on macrolide resistance. A large part of this study involved the determination of macrolide resistance rate in Portugal and the characterization of macrolide resistant isolates. The work presented here involved the determination of the macrolide resistance phenotypes and genotypes and its evolution throughout a large period of time, from 1998 to 2011. In order to identify the circulating clones in this period and to compare them with others in circulation in other European countries, we also performed the phenotypic and molecular characterization of the resistant isolates. In an attempt to find out if the macrolide resistant population was mirroring the general population, a study comparing both susceptible and resistant populations was performed, providing new insights about the dynamics of the resistant population.

Intrahost variation in infection

The incidence of genotipically or phenotipically different isolates of the same species in the same niche is well known for bacteria; a classic example is the co-

colonization with *S. pneumoniae*, or the carriage of multiple (generally two) different pneumococcus isolates [6, 27, 33]. In *S. pyogenes* there are few studies and contradictory results and we raised the question of whether the usual procedure in the microbiology laboratory (the isolation and subculture of one single colony to perform antimicrobial susceptibility testing and molecular characterization) was the most suitable to identify the real antimicrobial resistance rate. The fact that macrolide and tetracycline resistance genes are carried in mobile genetic elements, with the ability to be transferred between streptococcal species [19, 20, 24, 32] could contribute to this heterogeneity even in the same host. However, the results presented in chapter 2 of this thesis demonstrated that lateral gene transfer events, leading to the emergence of a resistant subset of isolates within the infecting population are not occurring in pharyngitis, at least in our collection. These results are in agreement with one study that included both carriage and infection isolates [29] but are contrary to another in which mixed infections with different isolates of the same genetic background were reported [40]. The difference between the latter study and the results presented here could be related to the fact that in our collection only one isolate presented *emm*3, which was the *emm* type expressed for by all the isolates that showed antimicrobial resistance or molecular differences. However, this situation seems to be unlikely, because we have previously demonstrated that although *emm*3 isolates were highly prevalent among the macrolide susceptible GAS population causing pharyngitis, they are rarely found among the resistant population. Nevertheless, our results seem to prove the adequacy of the single colony strategy commonly used in the microbiology laboratory in routine and epidemiological studies.

Epidemiology of macrolide-resistant GAS in Portugal

In Portugal, high rates of macrolide resistance in *S. pyogenes* causing tonsillo-pharyngitis were reported in the beginning of this study. In the first period analyzed here, between 1998 and 2003, macrolide resistance was one the highest in Europe (27%) and stable. However, this stability masked a complete inversion of the prevalence of the macrolide resistance phenotypes with the MLS_B phenotype that accounted for 85% of the isolates recovered in 1998 being expressed by only 23% of the resistant isolates in 2003.

This situation was very unusual, especially because is occurred in a very short period of time.

The frequent association of the dominance of the M phenotype with high macrolide resistance rates, reported in Spain [2], in Taiwan, the US and Canada [21, 22, 25] suggested a role for the higher genetic mobility of the elements carrying the resistance determinants conferring the M phenotype in the rapid dissemination of some successful clones, with a concomitant increase in the macrolide resistance rate [24]. However the situation reported in Portugal, as well as in Italy and France, was in sharp contrast: high rates of macrolide resistance were reported in populations with a clear dominance of the MLS_B isolates [3, 15]. Furthermore, in other European countries, a dominance of the MLS_B phenotype was accompanied by lower macrolide resistance rates (average 12%) [28]. All these countries present differences in the clonal composition of the populations, implying an important role for the circulating clones in the prevalence of both macrolide resistance rates and phenotypes.

At this point, it was known that in order to achieve a better characterization of the genetic lineages of *S. pyogenes*, other typing methods, such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST), had to be performed [7], but the fact that isolates presenting the M phenotype were resistant to SmaI digestion was hampering the comparison of the PFGE profiles. This resistance was proved to be due to the presence of a hybrid bacteriophage/transposon genetic element that encoded a methyltransferase that rendered DNA refractory to cleavage by the endonuclease SmaI [17]. Resistance to SmaI digestion was described frequentely, and to overcome this, a digestion with alternative enzymes such as SfiI was carried out [11, 14, 39]. However, this approach did not allow the direct comparison of M and MLS_B isolates, which from our point of view was very important. Given this, Cfr9I (a SmaI isosquizomer) was used and an UPGMA dendrogram could be generated including all the isolates, independently of their phenotypes. Using PFGE, it was possible to identify 8 major lineages, mostly defined by the same *emm* type, T type and ST, grouping more that 90% of the isolates in a population with high genetic diversity. All the genetic lineages were found to be described in other European countries, although in some cases the macrolide resistance rates and the prevalence of the macrolide resistance phenotypes showed significant differences, implying a role for other properties of these genetic lineages or differences in antimicrobial

consumption. The unusual situation documented in Portugal motivated the pursuing of these studies and with the inclusion of isolates recovered in the following 3 years it was possible to detect a decreasing trend in macrolide resistance that started in 1999. The fluctuations in the prevalence of both macrolide resistance phenotypes were still evident, although in the period 2004-2006, this decrease in macrolide resistance could be attributed mainly to a decrease in the prevalence of the M phenotype. Some differences were noted in the clonal composition of the population, but overall, the main genetic lineages identified had already been found in Portugal. The decrease in macrolide resistance still accompanied by fluctuations in the prevalence of the phenotypes in a population with high genetic diversity led to continued surveillance studies, especially because macrolide consumption, known to be a major driver of erythromycin resistance was one of the highest in Europe [1]. In spite of this, we reported a continuing decline in macrolide resistance from 2007 to 2011. Possible explanations for this unusual pattern can only be speculative. Macrolide consumption should not be the cause, although the data we refer to is the total consumption, without a differentiation between children and adults. If pediatric consumption was much lower, then the decrease in macrolide resistance could be a consequence of this difference, because the great majority of isolates included in this study were isolated from children. However, we have currently no means to find out the real pediatric consumption.

Tetracycline resistance is often referred as possible factor contributing to alterations in macrolide resistance rates [30], since the genetic elements responsible for resistance to both antibiotics are often carried together. In the subset of isolates included in this study, tetracycline resistance is, with only one exception, carried in some lineages of MLS_B isolates. Co-selection could have played a role in the decrease of macrolide resistance in 1999 to 2011, because a decrease in tetracycline consumption, concomitant to a decrease in tetracycline resistance was reported between 2000 and 2009 [31]. However, in that period, tetracycline consumption was much lower than erythromycin consumption; it would be less likely that tetracycline consumption would affect macrolide resistance than the contrary, although this is only speculative.

One possible explanation for the decrease in macrolide resistance is possible immunity developed by the human population against some of the antigens and M proteins displayed by *S. pyogenes*. The analysis of the clonal composition of the population in the

whole period studied in this thesis (1998-2011) (Figure 3.4.3, chapter 3.4) revealed large fluctuations of the main *emm* types, with waves of clones that, with few exceptions, are present in the population throughout the study period. It is possible, although speculative, that the immunity developed against some *emm* types can also confer protection against other *emm* types, as already demonstrated in GAS vaccine studies [13, 23], leading to a decrease in macrolide resistance if the circulating clones present immunologically cross-reacting M proteins.

Another possible explanation could be the existence of transmission differences between the different *emm* types, or genetic lineages, as already reported for *S. pneumoniae* [16, 37], where small but significant transmissibility differences were found between some serotypes. If *emm*11, more prevalent in the last years of the study would have had a smaller ability for host-to-host transmission, then its dominance among the macrolide resistance population would drive macrolide resistance down. On the contrary, if other *emm* types, like *emm*22, associated with high macrolide resistance in the early years of the study, would have an intrinsic feature conferring a higher transmissibility rate, than the successful spread of this clone would have the opposite effect on macrolide resistance. However, this is only speculative and could be unrelated to the main antigens, since the same genetic lineage (*emm*11-T11-ST403) was associated with an increase in macrolide resistance in Spain. However, other properties of this lineage can be different between the two countries, undetectable by the typing methods used in current GAS epidemiological studies.

Even considering all these possible explanations, it is still intriguing how in a context of high macrolide consumption, a high macrolide resistance rate was not sustained. It is particularly interesting how high intermediate-acting macrolides, known to select for the *erm*(B) gene, paralleled a decrease in the prevalence of MLS_B isolates, contrary to a previous study, that found that clarithromycin, an intermediate-acting macrolide was more effective in eradicating the *mef*(A) isolates than long-acting macrolides, such as azithromycin, thereby allowing the expansion of *erm*(B) carrying isolates [26]. According to the same study, the consumption of azithromycin would favor the emergence of M isolates, which in Portugal was only evident until 2003 although the use of long-acting macrolides was reported to be high during the whole study period [1].

All the genetic lineages identified among macrolide resistant GAS in Portugal had been previously described in other countries. Frequently, macrolide resistance genotypes associated to each major lineage are the same, which could suggest a geographic dissemination of a few resistant clones. However, independent acquisition of resistant genes by the prevalent genetic lineages, followed by local spread must also play a role, because it is also very common to find lineages with the same characteristics as the ones described here in susceptible isolates or in isolates presenting a different resistance conferring genotype, as is the case of *emm*12-ST36 isolates: in the period between 1998 and 2003, this lineage was mostly associated with the M phenotype and the *mef*(A) gene, while in 2004-2006, these isolates carried the *erm*(B) gene and presented the $cMLS_B$ phenotype.

Erythromycin-resistant and -susceptible GAS

In an effort to explain the origin of the macrolide resistant GAS in Portugal, a subset of susceptible and resistant isolates recovered between 2000 and 2005 was compared. At this point, it remained unknown whether the macrolide resistant clones circulating in Portugal were simply the reflection of the general population, emphasizing the role of the local acquisition of the resistant determinants followed by dissemination or the introduction of other successful resistant clones the from different locations.

Given the differences found and presented in chapter 4 of this thesis, reflected in the *emm* types and PFGE clones associated to each of the macrolide susceptible and resistant subsets, as well as in the diversity of the populations, the macrolide resistant population seems to have its own dynamics, rather than mirroring the behavior of the general population. Given this, it was suggested that horizontal gene transfer resulting in the acquisition of macrolide resistant determinants by the most frequent GAS lineages circulating is not frequent and the macrolide resistant clones described in Portugal are probably the result of the geographic dissemination of a few resistant lineages. The reasons why some *emm* types, with high occurrence in the susceptible population like *emm*3, *emm*6, *emm*87 and *emm*89, are persistently rare or even absent among the resistant subset remains unknown. In agreement to our results, a recent study addressing the global *emm* type distribution identified *emm*1, *emm*12, *emm*28, *emm*3, *emm*4, *emm*89 and *emm*6 as the

major *emm* types found in GAS populations in high-income countries [36] and all except *emm*3, *emm*89 and *emm*6 were previously identified as one of the most widely dispersed clones of macrolide resistant *S. pyogenes* [41]. At least for these *emm* types, it is not a question of being in the right place at the right time, as stated by the authors, and this seems to be also the case of *emm*94, which is absent from our collection, is widely distributed in macrolide resistant clones, but relatively rare throughout the world [36, 41]. The dominance of these *emm* types among macrolide susceptible or resistant populations could be related to lower or higher transmission rates of susceptible and resistant isolates of the same *emm* type, respectively, as already demonstrated for *S. pneumoniae* where serotype susceptible and resistant strains showed a different ability to be transmitted [16].

Scarlet fever GAS

The large collection of *S. pyogenes* recovered from pharyngitis and scarlet fever patients and contradictory reports of association with specific GAS exotoxins motivated the study presented in chapter 5 of this thesis. In Portugal, a large number of different genetic lineages was found in association with scarlet fever, contrary to the recent outbreak reports [8, 38]. Although not significant, it was possible to establish an association between some *emm* types and scarlet fever. Among the 11 exotoxins screened in this collection, only *ssa* was associated with scarlet fever in a univariate analysis; using a multivariate analysis approach, it was possible to associate also *speA* and *speC*. However, all these associations can be simply reflecting the high prevalence of specific genetic lineages among the general GAS population [34]. These isolates could also have some intrinsic feature providing enhanced potential for causing scarlet fever that is unrelated to their exotoxins gene content, making it difficult to reach definite conclusions. A recent study found that *ssa* and *emm*4 was significantly associated with pharyngitis [18], and results presented in chapter 4 of this thesis showed that *emm*4, *emm*3 and *emm*87 were statistically associated with macrolide susceptibility in the general GAS pharyngeal population. All these lineages carried *ssa*; however, *emm*3 isolates carried *speA*, but not *speC*, contrary to *emm*4 and *emm*87 isolates, suggesting that the presence of both exotoxins is not necessary to the pathogenesis of scarlet fever.

Concluding remarks and future perspectives

The work presented in this thesis provides new insights into the dynamics of macrolide resistant *S. pyogenes* population. The results presented here seem to point to a role for the natural fluctuations of macrolide resistance clones in the prevalence of macrolide resistance rates; even in the presence of a selective force such as high antimicrobial consumption, macrolide resistance rate reached the lowest level ever reported in Portugal. However, continued surveillance studies are needed; there are reports of increasing macrolide resistance rates even when antibiotic prescriptions showed a decreasing trend [9]. As macrolides represent a suitable alternative to penicillin in the treatment of GAS infections, the updated knowledge of resistance rates and phenotypes is useful in the empiric treatment of these infections. On the other hand, the molecular characterization of macrolide resistant isolates is important in the understanding of the dynamics of specific clones in close geographic areas and to evaluate the success of particular clones.

The comparison between macrolide resistant and susceptible populations presented in chapter 4 revealed that the most frequent genetic lineages found among macrolide resistant GAS isolates are not simply mirroring the general population, indicated by differences in the diversity of both populations, detected in all typing methods used. There are specific genetic lineages associated with each of the subsets of isolates, and in some cases, only the analysis by PFGE could differentiate macrolide resistant and susceptible isolates of the same *emm* types, like *emm*4 and *emm*1. Apart from being included in different PFGE clones and having different antimicrobial susceptibility, they share all the other molecular properties (T type, *emm* type and ST), which could suggest a common origin. However, other differences between the resistant and susceptible lineages, not detected by the methods used in the characterization of these isolates, could be present and responsible for this segregation. In this way, it would be interesting to perform whole genome sequencing of both macrolide susceptible and resistant isolates, to detect any additional differences between the two populations, other than the ones detected here.

The intriguing observation that some highly prevalent genetic lineages among the general population are rarely detected in resistant isolates, could be attributed to the

presence of a genetic barrier to the acquisition of macrolide resistance determinants, as already proposed for the M protein [10, 35]. It would be interesting to evaluate the transferability of the genetic elements carrying macrolide resistance genes, among different genetic backgrounds. If the macrolide susceptible genetic lineages are more resistant to the acquisition of those genetic elements, then this could be the explanation for their low prevalence among the macrolide resistant population. Moreover, these transferability experiments could be done in the presence of human pharyngeal epithelial cell lines, because a role for the host in the activation and transfer of some of these genetic elements was already suggested [4, 5].

Although the genetic elements found among the isolates causing scarlet fever in the recently described outbreaks were mostly absent from our collection, continuing surveillance is still needed, because the introduction of a highly successful clone could promote a rapid increase in GAS scarlet fever cases [8, 38]. In order achieve a better understanding of the differences between the scarlet fever and pharyngitis lineages, evident by a discrepancy in the diversity of both populations, the whole genome sequencing approach could also be useful in trying to identify molecular markers of this syndrome.

The observation that the single colony strategy remains suitable in the correct estimation of macrolide resistance rates, also potentially avoiding treatment failure, led us to believe that the results presented in this thesis did not provide an underestimate macrolide resistance. However, it would be interesting to see if there is some heterogeneity among the size and sequence of the M protein in the same host, and its implications for the interaction with the immune system.

The work presented in this thesis provided new insights into the dynamics of the GAS macrolide resistant population and in the identification of some potential markers for scarlet fever. However, some of the questions remained unanswered and others arose from the results presented here. Macrolide consumption does not seem to be the only driving force in determining macrolide resistance, but the reason for different dynamics in the resistant population are still unknown. Some genetic lineages were more frequently linked to resistance than others. Probably other molecular properties make these lineages more

prone to acquire and spread macrolide resistance, like a higher susceptibility to the acquisition of mobile genetic elements containing antimicrobial resistannce determining genes or others. Similarly, some genetic lineages were more prevalent in scarlet fever isolates than in pharyngitis isolates. Both populations showed different diversities, suggesting that one is not mirroring the other. The potential molecular markers, other than exotoxins or specific *emm* types, remain to be identified.

Some of the questions raised from the work presented in this thesis can be used to guide the future directions of this work. The use of other approaches, including next generation sequencing (NGS), will hopefully bring new insights into the dynamics of macrolide resistance in GAS, with an important contribution for the knowledge of such important human pathogen.

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