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Licenciado em Biologia

Evolution of genotypes of Group A streptococci from colonization and infection

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RESUMO

Streptococcus pyogenes é uma bactéria patogénica do Homem associada a uma variedade de infecções e doença, desde a pouco grave mas altamente prevalente faringite até a infecções extremamente severas, tais como a fasceíte necrosante e o síndroma do choque tóxico estreptocócico.

Este trabalho teve como objectivo o estudo de aspectos importantes e não explorados da epidemiologia, transmissão e evolução de *S. pyogenes* causadores de colonização e doença.

A amostra em estudo incluiu 1629 isolados de *S. pyogenes* associados a colonização e infecções. Destes 1629 isolados, 1026 foram recolhidos de 10578 exsudados da orofaringe de populações assintomáticas (crianças e adultos) durante 2000-2007 e 603 isolados foram provenientes de pacientes diagnosticados com infecções: 487 com amigdalite/faringite em 2000-2006, 72 com infecções de pele/tecidos moles em 1999-2005 e 44 com infecções invasivas em 1999-2005.

Este estudo revelou que os portadores saudáveis foram colonizados por uma população muito heterogénea de *S. pyogenes*. A taxa de colonização média foi maior entre as crianças em idade pré-escolar (0-6 anos) do que entre crianças com idade escolar (7-16 anos) e a frequência de colonização foi superior durante os períodos de inverno, o que sugere que o contacto entre crianças nas creches poderá favorecer o aumento de colonização por *S. pyogenes* em crianças saudáveis do pré-escolar.

Foi ainda observada uma elevada diversidade de estirpes de *S. pyogenes* associada a colonização de longo prazo e detectada co-colonização por várias estirpes de *S. pyogenes*.

Neste trabalho foi reportada, em portadores, a persistência de longo prazo da linhagem *emm*28/ST52, com reduzida resistência à bacitracina, a qual é prevalente na Europa. Adicionalmente, foi reportada pela primeira vez, a elevada resistência à bacitracina associada à linhagem *emm*74/ST120, a qual não era conhecida por incluir isolados resistentes à bacitracina.

Neste estudo foram reportadas, também pela primeira vez, inversões temporais de fenótipos de resistência a macrólidos em isolados de colonização, reforçando a importância da vigilância de portadores, pois estes podem ser indicadores do conjunto de isolados presentes na comunidade e que podem causar infecções. A elevada prevalência (>20%) dos genes de virulência *speC*, *prtF1* e *ssa* foi causada provavelmente por disseminação clonal (*speC*) ou por eventos de transferência génica horizontal (*prtF1* e *ssa*).

Neste trabalho foi observado que a taxa de susceptibilidade reduzida à ciprofloxacina foi ligeiramente inferior em isolados de colonização (4.3%) do que em isolados clínicos (6.0%). À excepção de um isolado, nos restantes foram identificadas mutações em *parC*-QRDR, originando as substituições amino-acídicas S79A (n=63) e D83G (n=2); a substituição D83G foi descrita pela primeira vez neste estudo e a associação entre isolados *emm*1 e reduzida

susceptibilidade a fluoroquinolonas foi também detectada pela primeira vez neste trabalho.

Foi observada uma maior frequência de genes de virulência em isolados associados a doença estreptocócica, tais como amigdalite/faringite, infecções de pele/tecidos moles ou doença invasiva, do que em colonização. Este estudo, em particular, contribuiu para um maior conhecimento dos factores de virulência de *S. pyogenes* que circulam neste país.

De entre os 1629 isolados, foram descobertos novos genótipos, tais como os subtipos *emm* 6.63, 28.9, 53.10, st4040.0 e stMrp6.0, assim como as sequências tipo ST380, ST397, ST398, ST401, ST402, ST427, ST428, ST429, ST430, ST431 e ST581.

Em conclusão, os resultados desta dissertação ampliam o nosso conhecimento sobre o estado de portador de *S. pyogenes*, a susceptibilidade a agentes antimicrobianos, epidemiologia molecular e virulência de isolados de *S. pyogenes* provenientes de colonização orofaríngica e infecções sintomáticas.

Palavras-chave: *Streptococcus pyogenes*, colonização, infecção, resistência antimicrobiana, virulência.

ABSTRACT

Streptococcus pyogenes is one pathogenic bacterium of humans and is associated with a wide variety of infections and disease states, ranging from uncomplicated but highly prevalent pharyngitis to extremely severe infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome.

This work aimed to study important and unexplored aspects of the epidemiology, transmission and evolution of *S. pyogenes* causing colonization and a wide range of diseases.

Our sample included 1,629 nonduplicated *S. pyogenes* isolates associated with colonization and infections. Out of the 1,629 isolates, 1,026 were recovered from 10,578 throat swabs of asymptomatic populations (children and adults) during 2000-2007 and 603 isolates were from patients diagnosed with clinical infections: 487 with tonsillitis/pharyngitis in 2000-2006, 72 with skin/soft tissue infections in 1999-2005, and 44 with invasive diseases in 1999-2005.

This study demonstrated that a very heterogeneous population of *S. pyogenes* colonized healthy carriers. The mean carrier rate was higher among pre-school children (0-6 years) than among school-aged children (7-16 years) and colonization frequency was higher during Winter periods, which suggests that the crowding of children in day-care centers may possibly increase the carrier rate in healthy pre-school children.

Moreover, it was also found that a high diversity of *S. pyogenes* strains was associated with long-term colonization, and it was detected co-colonization of the oropharynx by multiple *S. pyogenes* strains.

In this work, it was reported the long term persistence among carriers of the low-level bacitracin-resistant *emm*28/ST52 lineage, which is prevalent in Europe. It was also reported for the first time a high-level bacitracin-resistant isolate of the *emm*74/ST120 lineage, which was not previously known to include bacitracin-resistant isolates.

In this study were also reported for the first time temporal inversions of macrolide resistance phenotypes among colonization isolates, reinforcing the importance of surveillance of carriers, as they may be indicators of the pool of isolates circulating in the community that may cause infections. The high prevalence (>20%) of virulence genes *speC*, *prtF1* and *ssa* was probably caused either by clonal dissemination (*speC*), or to horizontal gene transfer events (*prtF1* and *ssa*).

In this work it was observed that ciprofloxacin-nonsusceptibility rate was slightly lower among colonization isolates (4.3%) than among the clinical isolates (6.0%). All but one ciprofloxacin-nonsusceptible isolates had *parC*-QRDR mutations generating the aminoacid substitutions S79A (n=63) and D83G (n=2); the ParC-D83G substitution was found for the first

time in this study and *emm*1 association with fluoroquinolone-nonsusceptibility was also first detected in this work.

It was observed a higher frequency of virulence genes among isolates from disease, such as tonsillitis/pharyngitis, skin/soft tissue infections or invasive disease, than among colonization. In particular, this study contributed to a better knowledge of *S. pyogenes* virulence factors that circulate in this country.

Among the 1,629 isolates, novel genotypes were discovered, such as *emm*-subtypes 6.63, 28.9, 53.10, st4040.0 and stMrp6.0, as well as sequence types ST380, ST397, ST398, ST401, ST402, ST427, ST428, ST429, ST430, ST431 and ST581.

In conclusion, the results from this dissertation extend our knowledge about the carrier state, the susceptibility to antimicrobial agents, molecular epidemiology and virulence of *S. pyogenes* isolates from oropharyngeal colonization and symptomatic infections.

Keywords: *Streptococcus pyogenes*, colonization, infection, antimicrobial resistance, virulence.

THESIS OUTLINE

The studies presented in this Doctoral Thesis focused on the antimicrobial susceptibility patterns, molecular epidemiology and virulence profiling of both colonizing- and infection-derived *Streptococcus pyogenes* isolates.

The order of presentation of each chapter in the present Doctoral Thesis does not necessarily reflect a chronological order, since some of the works described below were done simultaneously and the results obtained during one particular work would influence the progress of the other and vice-versa.

Chapter I consists of a general introduction and briefly describes the current state of the art of *S. pyogenes* species. Special attention was given to the general characteristics of the species, the carrier state and symptomatic infections, the most common virulence factors reported in this species, the resistance that antimicrobials confer to, the molecular tools used in *S. pyogenes* epidemiological studies, the *S. pyogenes* genome and vaccine candidates.

Chapter II reports important features of *S. pyogenes* oropharyngeal colonization, such as the clonal structure of sporadic and persistent strains, the long-term colonization status and the occurrence of co-colonization of the oropharynx by multiple strains.

Chapter III describes the molecular epidemiology, macrolide susceptibility and virulence profiles of *S. pyogenes* from healthy carriers.

Chapter IV presents the description of ciprofloxacin-nonsusceptible *S. pyogenes* isolates recovered from colonized and infected children, as well as the characterization of the associated clones and resistance mechanisms.

Chapter V describes the characterization of bacitracin-resistant *S. pyogenes* collected from oropharyngeal carriers and patients with diagnosed infections by the assessment of the putative variability of genetic backgrounds and of virulence genotypes of the isolates.

Chapter VI reports the screening for the presence of virulence genes in isolates from oropharyngeal colonization and symptomatic infections and the study of gene expression in selected isolates.

Chapter VII presents the major findings of this Thesis, which are highlighted and discussed. Also, future directions are suggested in this chapter.

ABBREVIATIONS

2YT – 2X Yeast-tryptone

A – Alanine

ABC – ATP-binding cassette

AP1 – Ancillary protein 1

AP2 – Ancillary protein 2

Arp – Immunoglobulin A receptor protein

ATP – Adenosine triphosphate

BHI – Brain-heart infusion

BP – Backbone protein

bp – Base pair

C – Colonization

CDC – Centers for Disease Control and Prevention

cDNA – Complementary DNA

CFU – Colony-forming units

CIP – Ciprofloxacin

CLSI – Clinical and Laboratory Standards Institute

$cMLS_B$ – Constitutive resistance to macrolides, lincosamides and streptogramins B

CO_2 – Carbon dioxide

CSO – Centro de Saúde de Oeiras

D – Aspartic acid

DCC – Day-care center

DEPC – Diethylpyrocarbonate

DID – Defined daily doses/1,000 inhabitants/day

DNA – Deoxyribonucleic acid

DNase – Deoxyribonuclease

dNTP – Deoxynucleotide triphosphate

DTT – Dithiothreitol

E – Glutamic acid

EDTA – Ethylenediamine tetraacetic acid

EL – Expression level

erm – Erythromycin ribosome methylation

ESAC – European Surveillance of Antimicrobial Consumption

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

FISH – Fluorescent *in situ* hybridization

G – Glycine

GAS – Group A *Streptococcus* or streptococci

gki – Glucose kinase

gtr – Glutamine transporter protein

GyrA – DNA gyrase subunit A

GyrB – DNA gyrase subunit B

IFN-γ – Gamma interferon

IL-1 – Interleukin-1

iMLS$_B$ – Inducive resistance to macrolides, lincosamides and streptogramins B

L – Leucine

LVX – Levofloxacin

Mb – Megabase pair

mef – Macrolide efflux

$MgCl_2$ – Magnesium chloride

MHC – Major histocompatibility complex

MIC – Minimum inhibitory concentration

MLS$_B$ – Co-resistance to macrolides, lincosamides and streptogramins B

MLST – Multilocus sequence typing

murI – Glutamate racemase

mutS – DNA mismatch repair protein

MXF – Moxifloxacin

N – Asparagine

NaCl – Sodium chloride

NK/ND – Not known/not done

NOR – Norfloxacin

NT – T nontypeable

O_2 – Oxygen

OC – Oropharyngeal colonization

OD – Optical density

P – Proline

PANDAS – Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections

ParC – Topoisomerase IV subunit C

ParE – Topoisomerase IV subunit E

PBP – Penicillin-binding protein

PBS – Phosphate buffer

PCR – Polymerase chain reaction

PFGE – Pulsed-field gel electrophoresis

PrtF1 – Protein F1

PrtF2 – Protein F2

PYR – L-pyrrolidonil-β-naphthylamide

QRDR – Quinolone resistance-determining region

recP – Transketolase

RFLP – Restriction fragment length polymorphism

RNA – Ribonucleic acid

RNase – Ribonuclease

rRNA – Ribosomal RNA

RT-PCR – Reverse transcriptase PCR

S – Serine

SAg – Superantigen

SfbI – Streptococcal fibronectin-binding protein I

SlaA – Streptococcal phospholipase A_2

SME-Z – Streptococcal mitogenic exotoxin Z

SOF – Serum opacity factor

Spd1 – Streptococcal phage DNase

SPE – Streptococcal pyrogenic exotoxin

SSA – Streptococcal superantigen

S/STI – Skin/soft tissue infections

ST – Sequence type

STSS – Streptococcal toxic shock syndrome

TBE – Tris-borate EDTA buffer

TCR – T-cell receptor

TE – Tris-EDTA buffer

T/P – Tonsillitis/pharyngitis

Tris – 2-Amino-2-hydroxymethyl-propane-1,3-diol

UP – Undecaprenol monophosphate

UPGMA – Unweighted pair-group method with arithmetic mean

UPP – Undecaprenol pyrophosphate

xpt – Xanthine phosphoribosyl transferase

Y – Tyrosine

yqiL – Acetyl-CoA acetyltransferase

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Chapter I

GENERAL INTRODUCTION

1. *Streptococcus pyogenes* or Group A *Streptococcus* (GAS)

1.1. General features

In this section we describe the general characteristics of the species, an overview of the identification methods used for differentiation of *S. pyogenes*, the niche of this pathogen and its host.

S. pyogenes is also known as beta-hemolytic Group A *Streptococcus* or Lancefield's Group A strep (GAS) (Facklam, 2002). It is a facultative anaerobic microorganism, catalase and oxidase negative, and its metabolism is fermentative. Individual cells have spherical shape and no motion. Colonies produce streptolysin-O, lysing completely the erythrocytes and forming large zones of beta-hemolysis (two or four times the diameter of the colony), when cultured on blood agar plate after 18 to 24 hours of incubation at 37ºC (see Figure 1.1) (Facklam, 2002). The cell wall of *S. pyogenes* contains an antigenic polysaccharide composed of N-acetilglucosamine linked to a rhamnose polimer backbone, whose serologic reactivity allows identifying primarily this species as Lancefield Group A (Cunningham, 2000). The true incidence of non-*S. pyogenes* GAS strains found in human infections, such as *S. dysgalactiae* subsp. *equisimilis* and *S. anginosus* group, is unknown, but from the information available at the Centers for Disease Control and Prevention (CDC) *Streptococcus* laboratory, these strains are not common (Facklam, 2002). Presumptive identification can also be made by bacitracin susceptibility or by the ability to enzymatically hydrolyse PYR (L-pyrrolidonil-β-naphthylamide). *S. pyogenes* strains are the only beta-hemolytic streptococci that are positive in both of these tests (Facklam, 2002). Other techniques have been used for *S. pyogenes* identification, including 16S rRNA sequencing and assessment of phenotypic characteristics by biochemical tests (Facklam, 2002), as well as fluorescent *in situ* hybridization (FISH) (Tajbakhsh *et al.*, 2011). Usually, *S. pyogenes* is a free-living organism; however, its ecological niche appears to be quite narrow, being limited to upper-respiratory tract mucosa, as well as skin. The only known natural reservoir of this pathogen is the human (Bessen and Hollingshead, 2000).

As it was referred to above, susceptibility to bacitracin remains to be used as a criterion for presumptive identification of *S. pyogenes*. However, resistance to this antimicrobial agent in *S. pyogenes* was previously described (Facklam and Washington II, 1991), raising questions concerning the reliability of this criterion. Resistance to bacitracin in *S. pyogenes* has been also frequently associated to constitutive resistance to macrolides, lincosamides and streptogramin B ($cMLS_B$ phenotype) (Malhotra-Kumar *et al.,* 2003; Mihaila-Amrouche *et al.,* 2004; Pérez-Trallero *et al.,* 2004; Pires *et al.*, 2009).

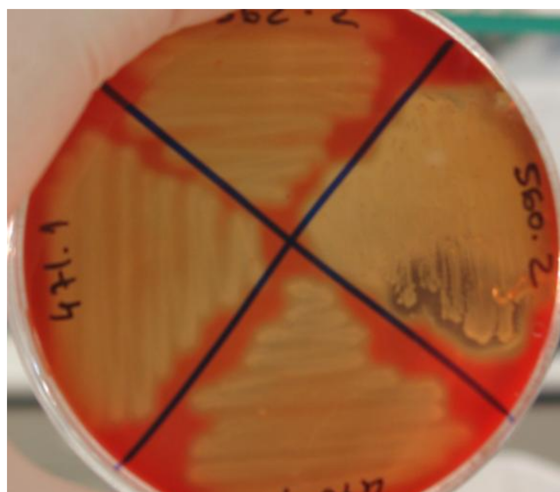

Figure 1.1. *Streptococcus pyogenes* strains grown on a blood agar plate with observation of beta-hemolysis (This study).

1.2. *S. pyogenes* carrier state: meaning and clinical relevance

Although it not be considered normal flora, *S. pyogenes* can colonize oropharyngeal respiratory tract without manifestation of clinical infection symptoms by the host (Cunningham, 2000). Colonization is also considered an infection; however the association between the microorganism and the host is commensal (Berkovitch *et al.*, 2002). The *S. pyogenes* carrier is an asymptomatic individual with a positive oropharyngeal swab culture and without serological response, or with a positive culture after completing the appropriate treatment with antimicrobial agents (Martin *et al.*, 2004). Pichichero and Casey (2003) showed that 7% of 140 children were *S. pyogenes* carriers after 10 to 21 days of treatment with an antimicrobial from macrolide class. These studies indicated that, although the disappearance (or attenuation) of the symptoms, the microorganism was not eradicated after the treatment with several antimicrobial classes (Martin *et al.*, 2004). Nguyen *et al.* (1997) demonstrated that asymptomatic individuals previously exposed to treatments for streptococcal infection can be colonized by new *S. pyogenes* strains (new clones). *S. pyogenes* can be spontaneously eradicated from asymptomatic individuals without antimicrobial therapy, suggesting that active immunization can eliminate bacteria from oropharyngeal flora. The re-emergence can arise from family contacts that coexist with sick individuals.

The *S. pyogenes* prevalence in the throat is known to be more common in school-aged children (5-21%) (Gunnarsson *et al.*, 1997). The carrier state has an important role in the dissemination of this bacterium via aerosol, especially among children at schools, day-care centers and at home. Since carriers could be the source of infection, the study of the prevalence of healthy *S. pyogenes* carriers and the molecular epidemiology of the isolates may improve understanding about the origin and spread of this pathogen, allowing for more successful

control measures (Kim, 2000; Durmaz *et al.*, 2003; Fazeli *et al.*, 2003). The genotypic comparison of *S. pyogenes* strains from asymptomatic carriers and from individuals with disease symptoms is also crucial for the understanding of global epidemiology of this pathogenic agent and from factors (from the host or from the bacterium) that control the development and severity of the disease (Efstratiou, 2000; Kim, 2000; Hoe *et al.*, 2002; Blandino *et al.*, 2011). An increase of epidemicity in an evolutive perspective can result from the acquisition of virulence or of antibiotic resistance genes by some strains, which could have selective advantage on others. On the other hand, the knowledge of hosts' population is important because susceptibility to disease can vary among individuals.

1.3. *S. pyogenes* symptomatic infections

S. pyogenes is considered to be the most pathogenic bacterium in the genus *Streptococcus*. The reason why this microorganism is a major public health concern is because is one of the most versatile and common human pathogens, causing a wide spectrum of diseases, ranging from mild infections to life-threatening systemic diseases (Facklam, 2002). Infections typically begin in the throat (examples are tonsillitis/pharyngitis or scarlet fever) or skin (impetigo). *S. pyogenes* may also cause disease in the form of nonsuppurative sequelae. These complications follow a small percentage of infections and include rheumatic fever, acute poststreptococcal glomerulonephritis and PANDAS (from "pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections"). Rheumatic fever is characterized by inflammation of the joints and/or heart following an episode of streptococcal pharyngitis. Acute glomerulonephritis, which is inflammation of the renal glomerulus, can follow streptococcal pharyngitis or skin infection (Efstratiou, 2000). PANDAS is characterized by the presentation of exacerbated neuropsychiatric symptoms following oropharyngeal infections caused by *S. pyogenes*. Among the invasive diseases caused by this pathogen, erysipelas and cellulitis are characterized by multiplication and lateral spread of *S. pyogenes* in deep layers of the skin. *S. pyogenes* invasion and multiplication in the fascia can lead to necrotizing fasciitis, a potentially life-threatening condition requiring surgical treatment. It can also lead to streptococcal toxic shock syndrome (STSS) (Cunningham, 2000). Although invasive diseases cause high morbidity and mortality (19-44%), its frequency of occurrence in Europe is very low (3/100,000) (Lamagni *et al.*, 2008). According to estimates, about 600 million cases of pharyngitis, 1.8 million new cases of severe disease, and 500,000 deaths occur each year worldwide (Carapetis *et al.*, 2005).

1.4. Virulence factors

The wide variety of disease syndromes caused by *S. pyogenes* is probably in part a reflection of the virulence gene products that are either secreted into the environment or localized on cell surface (Cunningham, 2000; Efstratiou, 2000). *S. pyogenes* utilises its many cell surface structures to adhere, internalise, and move across epithelia (Cunningham, 2000). A carbohydrate capsule composed of hyaluronic acid surrounds the bacterium, protecting it from phagocytosis by neutrophils. In addition, the capsule and several factors embedded in the cell wall, including M protein, lipoteichoic acid, and protein F (PrtF1/SfbI) facilitate attachment to various host cells (Figure 1.2.).

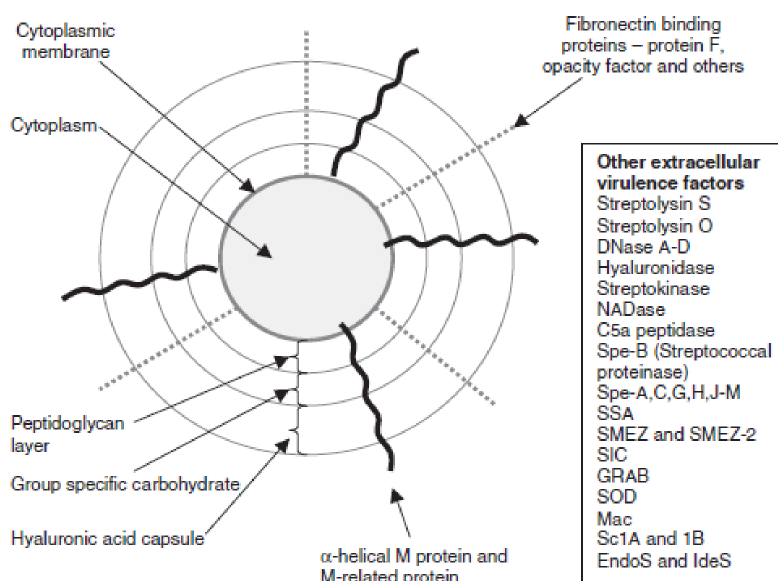

Figure 1.2. The basic outer cell antigenic structure of *S. pyogenes* (adapted from Steer *et al.*, 2007).

1.4.1. M protein and M-like proteins

The major virulence factor associated with *S. pyogenes* is the M-protein antigen. This surface antigen acts as an adhesin; promotes inflammation; impedes phagocytosis by binding complement control factors, fibrinogen, kininogen and also plasminogen; mediate the invasion of the cells and thus, surviving in the human host. The hypervariable N-terminal portion of this protein (see Figure 1.3) dictates the specific type of each antigen. M proteins are unique to each strain, and identification can be used clinically to confirm the strain causing an infection (Facklam, 2002). Strong sequence similarities to the C repeats (Figure 1.3) are found in Arp polypeptides (M-like proteins), but the so-called B repeats in Arp are not similar to those of several M serotypes (Nobbs *et al.*, 2009).

1.4.2. Superantigens

The superantigen (SAg) family can induce massive secretion of inflammatory cytokines, such as gamma interferon (IFN-γ), interleukin-1 (IL-1), and tumor necrosis factor-α. These molecules are stimulated by large numbers of T cells by the SAg cross-linking major histocompability complex (MHC) class II antigens and T-cell receptors (TCR) (Cunningham, 2000). Overproduction of these cytokines can lead to hypotension, fever, tissue damage, organ failure, and shock (Kotb, 1995). The discovery of novel SAg has accelerated with the completion of several genome sequencing projects and the total number now stands at 11: SPE-A, SPE-C, SPE-G, SPE-H, SPE-I, SPE-J, SPE-K, SPE-L, SPE-M, SSA, and the highly polymorphic SME-Z (Sriskandan *et al.*, 2007; Fraser and Proft, 2008).

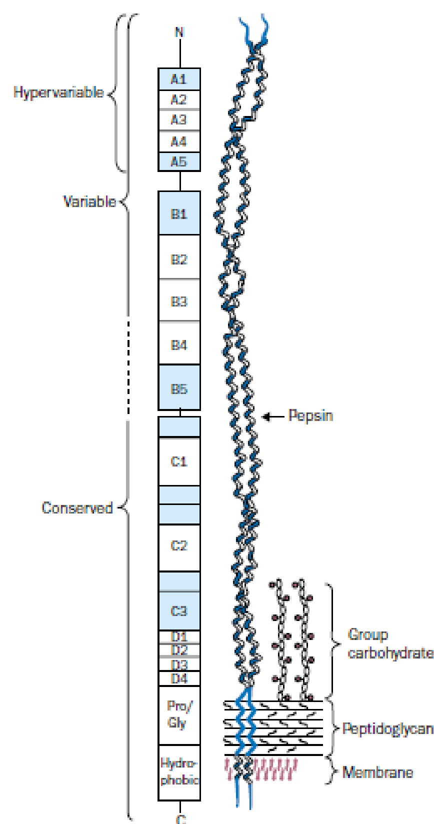

Figure 1.3. Diagrammatic representation of the M protein molecule on the cell surface of Group A streptococci (Adapted from Bisno *et al.*, 2003).

In the later 1990s, four novel SAg genes were identified, by mining the *S. pyogenes* M1 genomic database, at the University of Oklahoma. The genes for *speG*, *speH*, *speI*, and *speJ* were cloned and expressed in *Escherichia coli*, and the purified recombinant proteins showed the typical SAg features (Proft *et al.*, 1999; Proft *et al.*, 2001). The *S. pyogenes* genome project was completed in 2000, and since then three more SAg genes were discovered (Ferretti *et al.*,

2001; Proft *et al.*, 2003). One it is named *speK*, which is a pseudogene with an incomplete open reading frame (ORF) (Ferretti *et al.*, 2001). The other two are *speL* and *speM* genes and they are located on a mobile element (Φ*speL/M*) that enables gene transfer between individual isolates and between streptococci from different Lancefield groups (Proft *et al.*, 2003; Commons *et al.*, 2008).

SPE-A and SPE-C have been epidemiologically associated with the development of severe invasive diseases like STSS, or nonsuppurative sequelae such as rheumatic fever (McCormick and Schlievert, 2000). However, the *speA* gene was detected with comparable incidence among throat isolates and in invasive cases; in contrast, *speC* incidence was surprisingly higher among throat of paediatric populations than in invasive isolates (Creti *et al.*, 2005).

Most SAg-encoding genes are associated with bacteriophages, except for *speG*, *speJ* and *smeZ*, which are believed to be chromosomally encoded (Commons *et al.*, 2008). The gene distribution of superantigens has been used as an epidemiologic tool to explore genomic heterogeneity and the possible correlation between SAg gene content and clinical manifestation (Rivera *et al.*, 2006; Chang *et al.*, 2011).

The chromosomally encoded superantigens *speG* and *smeZ* have been described as the most prevalent, ranging from 84% to 100% of infection isolates (Proft *et al.*, 2000, 2003; Rivera *et al.*, 2006; Commons *et al.*, 2008). Isolates in which these superantigens were not detected were found to be restricted to certain *emm* types, e.g. *speG* was absent from *emm*4 isolates, suggesting that these isolates may contain an allele with mutations in the primer-binding sites (Commons *et al.*, 2008). The use of PCR with internal primers or DNA-DNA hybridization experiments could confirm the presence or absence of *speG* among isolates of *emm*4 type.

Streptococcal superantigen (SSA) is a toxin that has high similarity in sequence to SPE-A and is also phage-encoded. The toxin is usually produced by isolates of streptococci of the M protein capsular type M3 associated with STSS and by other M types of streptococci, but rarely by M1 isolates (McCormick and Schlievert, 2000; Commons *et al.*, 2008).

1.4.3. Streptococcal phospholipase A_2 (SlaA) and streptococcal phage DNase 1 (Spd1)

The *slaA* and *spd*1 genes are virulence factors also associated to phages: *slaA* is present in prophage Φ6180.2, being contiguous of *speK* gene; *spd1* is contiguous of *speC* gene in prophage Φ6180.1 (Green *et al.*, 2005b). The *slaA* gene encodes for an enzyme that hydrolyzes ester bonds of phospholipids (Nagiec *et al.*, 2004). The extracellular proteins encoded by *spd1* gene are involved in DNA destruction, however the role of these DNases was not be totally elucidated (Broudy *et al.*, 2002; Green *et al.*, 2005a).

1.4.4. Protein F1 (PrtF1) and other fibronectin-binding proteins

The fibronectin-binding proteins attach bacteria to the extracellular matrix, which acts as a bridge between streptococci and host cells. There are at least 11 fibronectin-binding proteins in *S. pyogenes*, including PrtF1, protein F2 (PrtF2), serum opacity factor (SOF), FbaA, and several M proteins (Nobbs *et al.*, 2009).

PrtF1, also known as SfbI (streptococcal fibronectin binding protein I), facilitates adherence to respiratory epithelial cells (Hanski and Caparon, 1992; Bisno *et al.*, 2003). Although M protein is known to mediate *S. pyogenes* adherence to skin keratinocytes, PrtF1/SfbI plays a major role in *S. pyogenes* adherence to cutaneous Langerhans cells (Okada *et al.*, 1994; Bisno *et al.*, 2003). Expression of PrtF1 is enhanced in an O_2-rich environment while that of M protein is greater at higher partial pressures of CO_2 (Caparon *et al.*, 1992; Bisno *et al.*, 2003). Thus, it was postulated that the organism displays PrtF1 on its surface when it wishes to adhere to the cutaneous surface but expresses M protein in deeper tissues where it is more likely to encounter phagocytic cells (Bisno *et al.*, 2003).

1.4.5. Pili

Pili are structures that extend 1 to 3 µm from the bacterial cell surface (Nobbs *et al.*, 2009). They are heteropolymeric structures consisting of a backbone protein (BP) and either 1 or 2 ancillary proteins (AP1 and AP2) covalently assembled and linked to the cell wall by a series of sortase-mediated transpeptidase reactions (Falugi *et al.*, 2008). In *S. pyogenes*, there are nine pilus islands so far reported that are inserted exclusively into a single highly variable genetic locus known as the fibronectin-binding, collagen-binding, T antigen (FCT) region (Falugi *et al.*, 2008) that forms part of the Lancefield T-serotyping system (Nobbs *et al.*, 2009). Pili play an important role in bacterial interaction with the human host. Streptococcal pili have recently been associated with the capacity to adhere to human epithelial cells and form biofilm, a process believed to be important in pathogenesis. For this reason, and because of their expression on the bacterial surface, pili have attracted interest as potential components of vaccines (Falugi *et al.*, 2008).

1.5. Antimicrobial therapy

Diverse classes of antimicrobials are used for the treatment of infections caused by *S. pyogenes*, such as streptococcal pharyngitis. Despite this microorganism is the most common cause of acute pharyngitis, only a small percentage of patients with these symptoms are infected with *S. pyogenes*. In this way, the prescription of antibiotics for the treatment of acute

pharyngitis must be done when is guaranteed that pharyngitis has bacterial origin, usually attributed to *S. pyogenes*, especially because the signs and symptoms of bacterial and viral pharyngitis are frequently overlapped (Bisno *et al.*, 2002).

The evaluation of antimicrobial susceptibility of all pathogenic microorganisms for infection treatment, *S. pyogenes* in particular, is crucial so that the therapy is appropriate, especially because it has been observed that antibiotics' consumption can be associated with the emergence of resistance in several countries (Bingen *et al.*, 2002; Bergman *et al.*, 2004).

The β-lactamic antibiotics are the most diversified and most frequently used antimicrobial class (Fluit *et al.*, 2001). This class includes penicillins (benzylpenicillin, amoxicillin, ampicillin, methicillin), cephalosporins (ceflacor, cefotaxime) and carbapenems (biapenem, ertapenem). These antibiotics act at penicillin-binding proteins (PBPs), which are involved in the bacterial cell wall synthesis. Bacterial resistance, in the most part of the cases, is due to the presence of β-lactamases (Fluit *et al.*, 2001), however in *Streptococcus pneumoniae* and in other streptococci, the emergence of resistance to β-lactamic antibiotics among natural populations is associated to the genesis of mosaic genes (Ferretti *et al.*, 2001), when segments of PBPs-encoding genes from susceptible strains are replaced by homologous blocks from resistant strains. These transfers are mediated by natural transformation with exogenous DNA and can cross the species boundary (Ferretti *et al.*, 2001). However, in the case of *S. pyogenes*, it was revealed the existence of two genes that encode for PBPs, both with low affinity and without homology comparing with other streptococcal corresponding genes (Ferretti *et al.*, 2001). By this way, acquisition of β-lactams resistance by homologous recombination with genetic material of other streptococcal species is unlikely. Besides that, and taking into account that there is no evidence that *S. pyogenes* is naturally competent for transformation, it is possible that β-lactams resistance in this bacteria has to appear *de novo* (Ferretti *et al.*, 2001). At the moment, it was not found any *S. pyogenes* strain that was resistant *in vitro* to β-lactams antibiotics, namely to penicillin (Ferretti *et al.*, 2001; Bingen *et al.*, 2004; Pires *et al.*, 2005), possibly due to the reasons referred to above and suggested by Ferretti and collaborators (2001). Other possible explanations for this remarkable state of continued susceptibility to penicillin are that β-lactamases may not be expressed or may be toxic to the organism and/or that low-affinity PBPs either are not expressed or render organisms nonviable (Horn *et al.*, 1998). In the last 50 years, penicillin is the most recommended antibiotic for the treatment of infections originated by *S. pyogenes*, due to its proven effectiveness, safety, short activity spectrum and low cost (Bisno *et al.*, 2002). However, penicillin treatment can fail in some cases, possibly due to the production of β-lactamases by other microorganisms from oral flora; so, a good choice for the empirical treatment of *S. pyogenes* will be the use of combination amoxicillin/clavulanic acid (Gerber *et al.*, 1999; Cunningham, 2000). In patients who are allergic to penicillins, macrolides (including erythromycin, azithromycin and clarithromycin) and lincosamides (including

clindamycin) seem to be adequate choices for the treatment of streptococcal infections (Bisno *et al.*, 2002).

1.5.1. Macrolides, lincosamides and streptogramins

1.5.1.1. Structure and mechanisms of action

The macrolides are a group of antibiotics that have a large lactone ring structure. These may be 14- (like erythromycin, see Fig. 1.4A, and clarithromycin), 15- (like azithromycin, see Fig. 1.4B) or 16-membered (like josamycin, see Fig. 1.4C) rings. These are relatively nontoxic antibiotics, most active against Gram-positive bacteria (Roberts *et al.*, 1999). Macrolides inhibit protein synthesis by stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation (Weisblum, 1995). This results in chain termination and a reversible stoppage of protein synthesis (Roberts *et al.*, 1999). The lincosamide antibiotic lincomycin and its semi-synthetic derivative clindamycin (see Fig. 1.4D) have a similar mode of action.

The streptogramins fall into two groups, A and B. Streptogramin belonging to Group A has a large nonpeptide ring, which is polyunsaturated. Streptogramins related to streptogramin B are cyclic peptides (see Fig. 1.4E). They differ in their modes of action although both inhibit bacterial protein synthesis. Group A streptogramins distort the ribosome to prevent binding of the tRNA; Group B streptogramins are thought to block translocation of the growing peptide.

1.5.1.2. Resistance mechanisms

Resistance to macrolides in *S. pyogenes* can be mediated by three different mechanisms: target site modification, active efflux and rRNA 23S or ribosomal proteins mutations. The modification of the target site is based in dimethylation N^6 of an adenine residue of rRNA 23S through the action of an enzyme family encoded by *erm* (“erythromycin ribosome methylation”) genes class. The methylation seems to induce a conformational alteration among 50S subunit of the ribosome, leading to a reduced affinity and to co-resistance to macrolides, lincosamides and streptogramin B (MLS_B antibiotics), whose binding sites probably overlap. The MLS_B resistance can be expressed constitutively ($cMLS_B$ phenotype) or can be induced ($iMLS_B$ phenotype) and it is mediated by two classes of methylase-encoding genes, the *erm*(B) and *erm*(A) [which includes the *erm*(TR) subclass)]. The induction is stimulated by 14- and 15-membered macrolides that are efficient inducers of methylase synthesis (Leclercq and Courvalin, 1991; Sutcliffe *et al.*, 1996; Giovanetti *et al.*, 2002).

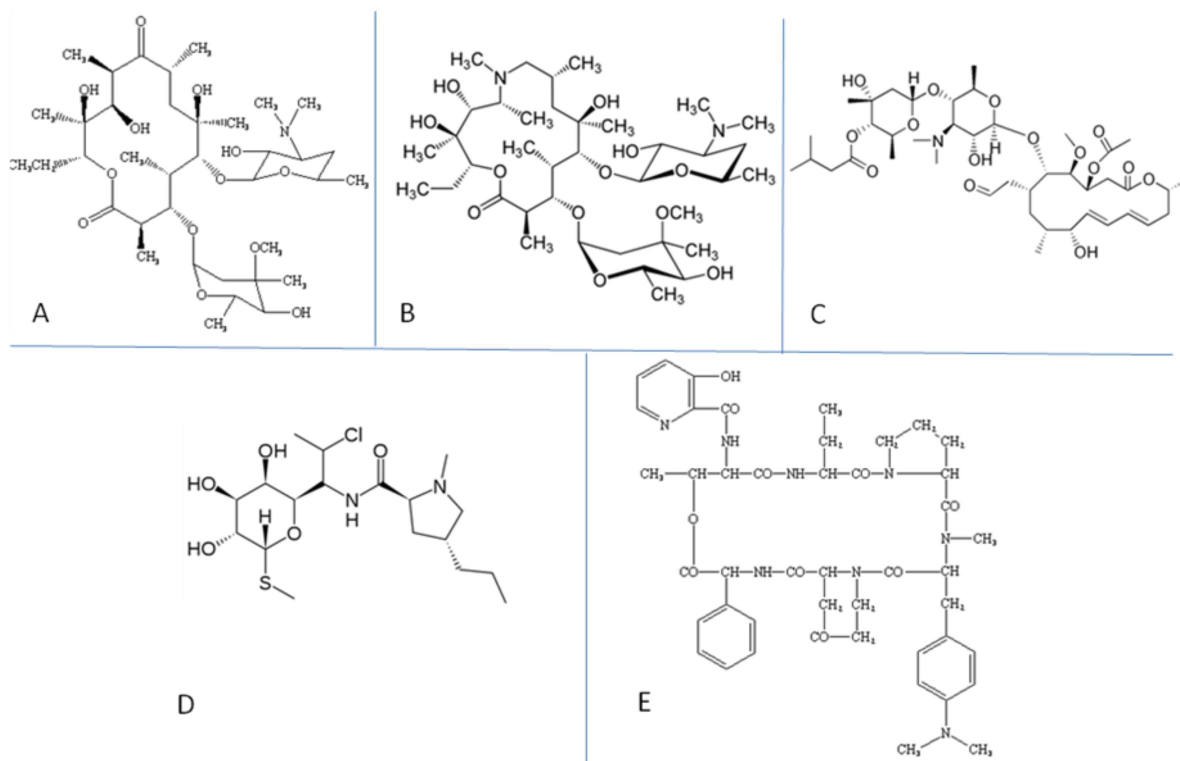

Figure 1.4. Examples of macrolides, lincosamides and streptogramins chemical structures: A- Erythromycin (14-membered macrolide); B- Azithromycin (15-membered macrolide); C- Josamycin (16-membered macrolide); D- Clindamycin (lincosamide); E- Streptogramin B. Adapted from http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/antibiotics/protein.html, http://www.antibioticslist.com/azithromycin.htm, http://www.antibioticslist.com/josamycin.htm and http://www.antibioticslist.com/clindamycin.htm.

The active efflux of antibiotic is mediated by hydrophobic membrane proteins, encoded by *mef*(A) ("macrolide efflux") resistance gene. A small portion of strains carry different *mef* subclasses, such as *mef*(E) and *mef*(I) (Del Grosso *et al.*, 2011). These proteins use the proton energy in order to pump out the antibiotic, maintaining its intracellular concentration low and consequently, the ribosome free of its activity. The mechanism occurs without antibiotic and its target modification and became the strains resistant to 14- and 15-membered macrolides, but susceptible to 16-membered macrolides, to lincosamides and to streptogramin B (Roberts *et al*, 1999). This resistance phenotype is named M phenotype.

The third resistance mechanism was described for *S. pyogenes* by Malbruny *et al.* (2002), and it was previously observed among *Streptococcus pneumoniae* as associated to a mutation in V domain of rRNA 23S. In a certain strain, this mutation originated cross-resistance to azithromycin, clindamycin and streptogramin B (MLS_B resistance), however the strain remained susceptible to erythromycin. The same authors observed the occurrence of a mutation among ribosomal protein L4 among one strain that was resistant to azithromycin and susceptible

to erythromycin, clindamycin and streptogramin B (M phenotype). Mutations in the gene that encodes this protein had been previously detected in *S. pyogenes* by Bingen *et al.* (2002). It was also described by Jalava *et al.* (2004) one *S. pyogenes* strain that was resistant to macrolides, lincosamides and streptogramin B, but did not present any macrolide resistance gene, but had a mutation in rRNA 23S in a different location as that described by Malbruny *et al.* (2002). This strain presented the $cMLS_B$ phenotype, similarly with strains that constitutively expressed the *erm*(B) genes.

1.5.2. Fluoroquinolones

1.5.2.1. Structure

The fluoroquinolones are synthetic antibiotics that belong to quinolones family. They are modified molecules that contain one or more fluorine atoms as well as other chemical alterations (Ball, 2000) (see Figure 1.5). Differences in the *in vitro* activity of the quinolones primarily form the basis of their classification in four generations, and these differences are based on antibacterial activity and potency against pneumococci and anaerobic organisms (Andriole, 2005). The first generation quinolones (nalidixic acid, oxolinic acid, cinoxacin, piramidic acid, pipemidic acid, and flumequine) exhibit an excellent activity against aerobic and Gram-negative bacteria. The second generation quinolones include norfloxacin, ciprofloxacin, ofloxacin, levofloxacin, enoxacin, flexacin, lomefloxacin, pefloxacin, and rufloxacin (Andriole, 2005). They were introduced when norfloxacin was synthesized by adding a fluorine atom in C-6 carbon and a cyclic piperazine diamine in C-7 carbon (Andriole, 2005). These changes added antimicrobial activity against aerobic Gram-positive bacteria and improved activity against Gram-negative bacteria, when compared with first generation quinolones. The third generation fluoroquinolones, such as grepafloxacin, gatifloxacin, sparfloxacin, temafloxacin, tosufloxacin, or pazufloxacin, are very efficient against Gram-positive bacteria, in particular against pneumococci, and they also had good activity against anaerobic bacteria. The fourth generation fluoroquinolones (trovafloxacin, clinafloxacin, sitafloxacin, moxifloxacin, and gemifloxacin) had potent activity against anaerobic bacteria and increased activity against pneumococci (Andriole, 2005).

1.5.2.2. Use of fluoroquinolones

Fluoroquinolones are used for the treatment of diverse infections in adults, namely urinary tract infections, skin infections, sexually transmissible infections, and lower respiratory

tract infections (Andriole, 2005). These antibiotics are not recommended for the treatment of infections among children because of high risk of tendon damage (Gendrel *et al.*, 2003).

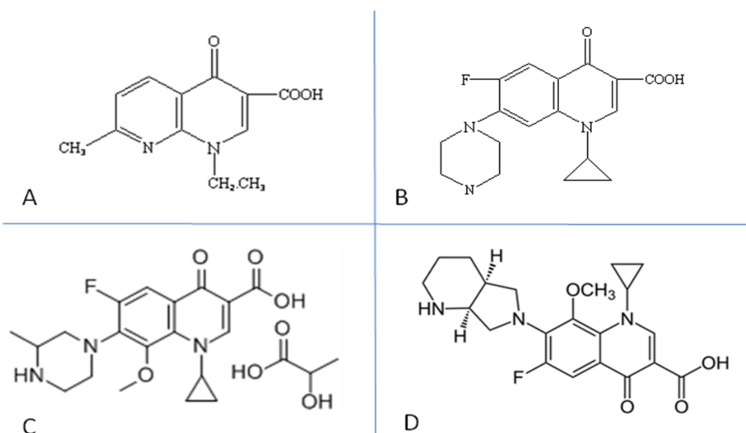

Figure 1.5. Molecular structures of quinolones: A- Nalidixic acid (first generation quinolone); B- Ciprofloxacin (second generation quinolone); C- Gatifloxacin (third generation quinolone); D- Moxifloxacin (fourth generation quinolone).
Adapted from http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/antibiotics/dna.html, http://www.chemicalbook.com/ProductChemicalPropertiesCB8227559_EN.htm and http://www.antibioticslist.com/moxifloxacin.html.

1.5.2.3. Mechanisms of action

The bactericidal power of fluoroquinolones is originated by inhibition of DNA replication and transcription, targeting two cellular enzymes: DNA gyrase and topoisomerase IV. The DNA gyrase, that originates DNA's negative supercoiling, is constituted by two GyrA and two GyrB subunits, which are encoded by *gyrA* and *gyrB* genes, respectively, the preferable target of fluoroquinolones in Gram-negative bacteria. Topoisomerase IV separates DNA chains during replication and cell division, and is constituted by ParC and ParE subunits, which are encoded by *parC* and *parE* genes, respectively, the preferable target among Gram-positive bacteria (Perichon *et al.*, 1997; Alonso *et al.*, 2002; Jacoby, 2005).

1.5.2.4. Resistance mechanisms

Fluoroquinolone resistance is mainly caused by point mutations in the target-encoding genes (*gyrA*, *gyrB*, *parC* and *parE*). Mutations tend to cluster in a defined region of these genes called the quinolone resistance-determining region (QRDR). Fluoroquinolone resistance appears to occur stepwise, with moderate levels of resistance arising from a single mutation in the primary target of the drug (topoisomerase IV, *parC* gene) (Alonso *et al.*, 2005; Orscheln *et al.*, 2005; Pires *et al.*, 2010). A higher level of resistance is reached by the accumulation of

additional mutations in the secondary target (DNA gyrase, *gyrA* gene) (Orscheln *et al.*, 2005). The mutations occur most frequently in codons S79 for ParC and S81 for GyrA (Yan *et al.*, 2000; Richter *et al.*, 2003). Recently, mutations in codons D83 for ParC (Pires *et al.*, 2010) and E85 for GyrA (Arai *et al.*, 2011) were described. Fluoroquinolone efflux by a reserpine-sensitive pump is also a common resistance mechanism to fluoroquinolones in *Streptococcus pneumoniae* (Brenwald *et al.*, 1998) and viridans group streptococci (Ferrándiz *et al.*, 1999), although this mechanism still remains to be demonstrated for *S. pyogenes*.

1.5.3. Tetracycline

Tetracycline is not recommended for therapy of upper respiratory tract infections originated by *S. pyogenes*. However, the selective pressure derived from its use in the treatment of other human and animal infections can have contributed for the worldwide emergence of resistance to this antibiotic among isolates of *S. pyogenes* (de Melo *et al.*, 2003). The transfer of genes from animals to humans through food is a possible explanation for the emergence of resistance (Nielsen *et al.*, 2004). Tetracycline resistance levels among enterococci are high in both humans and their feeding animals. As the horizontal transfer of tetracycline resistance genes from enterococci to streptococci has been demonstrated (Nielsen *et al.*, 2004), this transfer mediated by transposons is a real possibility in the oral cavity. In the transfer of tetracycline resistance genes from enterococci, the oral flora can also be used as a transition state because both *tet*(M) and *tet*(O) genes have been found in several bacterial species from oral flora (Nielsen *et al.*, 2004). The existence of *S. pyogenes* strains resistant to tetracycline contributes to the importance of including this antibiotic in antibiotyping studies.

Tetracycline is included in a group of antiobiotics with wide spectrum of activity and low toxicity. These antibiotics penetrate the bacterial cells by passive diffusion and bind to the 30S subunit of the ribosome, inhibiting the protein synthesis by blocking the connection between the aminoacil-tRNA and the ribosome A place. Several tetracycline resistance genes are described for *S. pyogenes*, such as *tet*(K), *tet*(L), *tet*(O) and *tet*(T), being prevalent the *tet*(M) gene (Shlaes, 2006). Some of these genes are often associated to conjugative transposons, which in part can explain their wide distribution among bacterial species, as well as their association with other antibiotic resistance genes, particularly the association between *tet*(M) and *erm*(B) genes (Fluit *et al.*, 2001). There are three known tetracycline resistance mechanisms: enzymatic inactivation of the antibiotic, antibiotic efflux by protons antiporte [*tet*(K) and *tet*(L)], and protection of the ribosomes by the production of one protein which interacts with the ribosome allowing the protein synthesis autonomy in the presence of the antibiotic [*tet*(M) and *tet*(O)] (Clermont *et al.*, 1997; Fluit *et al.*, 2001).

1.6. Epidemiology of *S. pyogenes*

In this topic the most common methods used in epidemiological studies of *S. pyogenes* will be presented: M-typing, T-typing, *emm*-typing, *sof*-typing, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). It is important to refer that most studies on the epidemiology of *S. pyogenes* are based on the analysis of antimicrobial resistance patterns (Cocuzza *et al*., 1997; Descheemaeker *et al*., 2000; Cresti *et al*., 2002), T-typing (Beall *et al*., 1997; De Azavedo *et al*., 1999; Melo-Cristino *et al*., 1999; Bingen *et al*., 2000, Pires *et al*., 2005), and *emm*-typing (Tyrrell *et al*., 2010; Bahnan *et al*., 2011; Chen *et al*., 2011), although resistance frequencies and phenotypes vary geographically.

M-typing - In 1928, Rebecca Lancefield published a method for serotyping *S. pyogenes* based on its M protein (Facklam, 2002). M type-specific antisera are used in a precipitin reaction against *S. pyogenes* acid extracts. Currently, there are approximately 90 validated and specific M types. There are some problems associated with M serotyping such as limited availability of M typing sera, the production of M sera is laborious, expensive, and only available to a few centers, difficulty in interpretation, newly encountered M types overtime and an increased frequency of nontypeable isolates (Neal *et al*., 2007). In the United States and Europe, it was found a high prevalence of M1 and M3 serotypes among invasive disease (Efstratiou, 2000). Type M28 strains also are common causes of invasive infections, as well as pharyngitis in many countries (Green *et al*., 2005b).

T-typing - In 1946, Rebecca Lancefield described the serologic classification of *S. pyogenes* isolates based on their surface T antigen. Typing of the T protein uses polyvalent pooled and monovalent antisera in a slide agglutination test. In *S. pyogenes*, type-specific T-protein antigens are basic markers for typing and can be divided into approximately 30 different T-types. Like M serotyping, T-serotyping have the same associated problems and a careful interpretation by experienced staff is needed. A high number of nontypeable isolates have been reported (Neal *et al*., 2007). The T-protein serotyping and its comparison with *emm* types provide additional information for strain identification (Johnson *et al*., 2006).

emm-typing - In recent years, an alternative system called *emm*-typing has been developed, which uses the sequence of the hypervariable region of the gene that encodes the M-protein (Beall *et al.,*1996). The method and a public database are of public access at The Centers for Disease Control and Prevention (CDC), *Streptococcus* Laboratory (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). The definition of an *emm*-type sequence is based upon the identity of 180 bases at the 5' terminal end of the hypervariable portion of the *emm* gene (see Fig. 1.3). Determination of the *emm* gene sequence has become a widely used alternative to M typing for GAS characterization, and there are currently 124 officially designated *emm* types (*emm*1 through *emm*124) (Last update: September 15, 2011).

The *emm*-types are further divided into subtypes that are explicitly based on minor sequence variation within the type specific hypervariable region of the gene. The website curator maintains the *S. pyogenes emm* sequence database with of all validated M and *emm* types, as well as provisional types and subtypes yet to be officially designated. In most cases the *emm* type reflects the M-protein serologic type (Facklam, 2002). Some restricted *emm*-types, such as *emm*1 and *emm*3, were more representative in invasive strains (Creti *et al.*, 2005), whereas *emm*12 was more common among asymptomatic carriers (Blandino *et al.*, 2011).

sof sequence typing - *S. pyogenes sof* (serum opacity factor) is approximately a 1000 residue cell-surface-bound apoproteinase named for its property of rendering various opaque sera. s*of* is a virulence factor since it has fibronectin-binding activity that resides in a relatively short C-proximal domain. *sof* gene detection and sequencing are based upon PCR and sequence analysis of a variable-length 450-650 bp PCR fragment using methods and primers decribed in Beall *et al.*, 2000. T-pattern and *sof* information about a *S. pyogenes* strain, especially when combined with knowledge of M or *emm*-type, provides an important link to information from studies published over many decades, when the serological methods were primary available tools (Johnson *et al.*, 2006).

Multilocus sequence typing (MLST) is a method based on nucleotide sequence of internal fragments of seven housekeeping genes (highly conserved genes) that are assumed to be neutral in their genetic variation (www.mlst.net). This allelic profile (a sequence of 7 numbers – one number for each allele and in a specific order: *gki*, *gtr*, *mur*I, *mut*S, *rec*P, *xpt* and *yqi*L) defines the sequence type (ST). The STs provide unambiguous results that are easily portable and a central database (http://spyogenes.mlst.net/) allows for comparison of results obtained in different laboratories (Enright *et al.*, 2001; Doktor *et al.*, 2005). Until September 15, 2011, a total of 624 STs were published at the international database. Also, it is considered to be the method of choice for global epidemiological studies and to evaluate the bacterial population structure and evolution by identifying lineages and clonal complexes.

Pulsed-field gel electrophoresis (PFGE) has been the gold standard method used to assess strain similarity in epidemiologic studies (Tenover *et al.,* 1995; Cocuzza *et al*., 1997; Haukness *et al*., 2002). However, it is a method particularly useful for short-term epidemiological studies or outbreak situations and not for global epidemiological studies. Several authors also consider that PFGE, like other methods based on DNA-band analysis, is not reproducible among different laboratories and band-patterns are difficult to interpret.

Although the advantages of sequence-based methods, *S. pyogenes* virulence has been related to the presence of phages and to horizontal transfer. This highlights the notion documented by several authors that PFGE may be more discriminatory than sequence-based methods (Bahnan *et al.*, 2011), since phage insertions can alter band positions in an agarose gel and, consequently, create more diversity within PFGE types (Carriço *et al.*, 2006).

1.7. *S. pyogenes* genome

To date, the genome sequences of 15 strains of *S. pyogenes* have been determined, including strains that were associated with various clinical conditions and representatives of the following ten serotypes: M1, M2, M3, M4, M5, M6, M12, M18, M28 and M49; the genomes of two separate strain isolates have been determined for serotypes M1, M3, M12 and M49. The genome is a circular chromosome between 1,3 and 1,9 Mb (Table 1.1). The genomes share 1.7 Mb of closely related genetic material (Beres *et al.*, 2002; Nakagawa *et al.*, 2003; McShan *et al.*, 2008), and phages, phage-like elements, and insertion sequences are the major sources of variation between the genomes (Smoot *et al.*, 2002). Genetic variation is essential to survival for all organisms (McShan *et al.*, 2008). These sequenced strains have genes encoding a novel array of prophage virulence factors, cell-surface proteins, and other molecules likely to contribute to host-pathogen interactions. Genetic relationship between strains causing invasive disease episodes to strains of the same serotype recovered from asymptomatic carriers was not examined yet. Importantly, it is not known whether strains cultured from asymptomatic carriers differ in virulence compared to invasive isolates (Beres *et al.*, 2006).

Table 1.1. List of sequenced *S. pyogenes* strains.

Strain	Size (bp)	M type	No. of prophages	Accession no.	Reference
SF370	1,852,441	1	4	AE004092	Ferretti *et al.*, 2001
MGAS5005	1,838,554	1	3	CP000017	Scott *et al.*, 2008
MGAS10270	1,928,252	2	5	CP000260	Beres *et al.*, 2006
MGAS315	1,900,521	3	6	AE14074	Beres *et al.*, 2002
SSI-1	1,894,275	3	6	BA000034	Nakagawa *et al.*, 2003
MGAS10750	1,937,111	4	4	CP000262	Beres *et al.*, 2006
Manfredo	1,841,271	5	5	AM295007	Holden *et al.*, 2007
MGAS10394	1,899,877	6	8	CP000003	Banks *et al.*, 2004
MGAS2096	1,860,355	12	2	CP000261	Beres *et al.*, 2006
MGAS9429	1,836,467	12	3	CP000259	Beres *et al.*, 2006
MGAS8232	1,895,017	18	5	AE009949	Smoot *et al.*, 2002
MGAS6180	1,897,573	28	4	CP000056	Green *et al.*, 2005b
NZ131	1,815,783	49	3	CP000829	McShan *et al.*, 2008
M49 591	1,327,684	49	na	AAFV01000000	Unpublished
ATCC 10782	1,839,847	na [a]	na	AEEO01000000	Unpublished

Legend: [a] – not available.

1.8. Vaccine candidates

Because of the high colonization rates (for example 32%, Martin *et al.*, 2004) and increased frequency in some countries of severe infections rates caused by this pathogen, and concerning that penicillin-resistant strains may emerge, there is a strong incentive to develop a safe and effective vaccine against *S. pyogenes*. One of the benefits of a successful vaccination scheme would be the reduction of streptococcal colonization in general, thus reducing the total number of these pathogens in the population. Since the main reservoir for *S. pyogenes* for most streptococci-related illness is the human oropharynx, reducing the carriage could have a profound impact on the dissemination of streptococci in the environment and thus a significant reduction in streptococcal disease in general.

The M protein has been a prime vaccine candidate to prevent Group A streptococcal infections since Lancefield showed clearly that M protein-specific human and animal antibodies have the capacity to opsonize streptococci in preparation for phagocytic clearance. A type-specific vaccine necessary to protect against a streptococcal infection would require a multivalent antigen corresponding to stable immunodeterminants on serotypes that together account for the majority of the isolates prevalent within the population at a given time (Fischetti, 2000). Attempts to develop an M protein-based vaccine have been hindered by the fact that some M proteins elicit both protective antibodies and antibodies that cross-react with human tissues. New molecular techniques have allowed the previous obstacles to be largely overcome. A 26-valent vaccine has successfully completed a phase I/II clinical trial involving adults and was proposed, suggesting that it could have significant impact on the overall burden of streptococcal disease (McNeil *et al.*, 2005).

Although promising, these multivalent M protein vaccines will not provide immunity against infections caused by strains of all M serotypes because of the variable nature of this protein. As consequence of these considerations, eight non-M protein vaccines has been in development, such as Group-A carbohydrate, C5a peptidase (ScpA), cysteine protease (SpeB), binding proteins like fibronectin, opacity factor, lipoproteins, Spes (superantigens) and streptococcal pili (Guilherme *et al.*, 2009). However, no vaccines containing these antigens have reached clinical trials (Steer *et al.*, 2009).

1.9. Objectives of the research

This Thesis was designed to study important and unexplored aspects of the epidemiology, transmission and evolution of *Streptococcus pyogenes* causing colonization and a wide range of disease syndromes.

Chapter II

AN EIGHT-YEAR SURVEILLANCE STUDY (2000-2007) OF OROPHARYNGEAL COLONIZATION IN PORTUGAL AND CHARACTERISTICS OF LONGITUDINAL OROPHARYNGEAL ASYMPTOMATIC COLONIZATION AND OF MULTICOLONIZATION BY DIFFERENT STRAINS OF *STREPTOCOCCUS PYOGENES*

Part of these results were presented at the 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), May 16-19, 2009, Helsinki, Finland; at the national meeting of microbiology and biotechnology (Congresso Nacional de Microbiologia e Biotecnologia) MicroBiotec09, November 28-30, 2009, Vilamoura, Portugal; and at the 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), April 10-13, 2010, Vienna, Austria.

ABSTRACT

During 16 periods in 2000-2007, oropharyngeal samples were taken from different populations: 6,965 from children (0-6 yrs) in day-care centers (DCCs), 2,337 from school-aged children (7-16 yrs) and 1,276 from adults (1,169 school staff and 107 family members). Between 2000 and 2004, 7,894 oropharyngeal samples were taken during 12 sampling periods in October, February and May of each year from 3,900 healthy individuals. Bacterial identification was carried out by standard methods. The total number of isolates from the longitudinal colonization study was 772 (carriage rate 9.8%) out of which 243 (31.5%) were from 113 (2.9%) recurrent carriers - defined as colonized more than once during 2 to 12 sampling periods. All beta-hemolytic colonies identified as *S. pyogenes* that were picked from each of the primary plates containing the swabs of 23 participants, out of 865 (2.7%) persons sampled in one sampling period, were considered as causing co-colonization or multicolonization of the same niche. Resistance to macrolides and clindamycin was evaluated by disk diffusion and minimal inhibitory concentrations using E-tests. Clones of all resistant and a subset of susceptible isolates were defined by pulsed-field gel electrophoresis (PFGE) and further characterized by serotyping for T antigen (T-typing), and by sequencing for assignment of *emm*-types and multilocus sequence types (ST). A total of 1,026 GAS were isolated (9.7%) from the point prevalence study. Oropharyngeal colonization (OC) in younger children was higher (11.6%) than among older than 6 years (7.8%), and in adults was higher among family members (8.4%) than among school staff (2.6%). OC rates varied with DCC (min. 0%; max. 49%). Higher OC rates (>10%) were usually detected during winter periods (17% in 2001, 13% in 2002/03, 12% in 2007), and occasionally, during autumn of 2001-2003 (11%-15%) and spring of 2001 and 2004 (15% and 18%, respectively). Resistance to macrolides was 10% in 2000-02, 28% in 2003, 20% in 2004, 3% in 2005, 14% in 2006 and 10% in 2007. Clindamycin resistance was lower than 10%, except in 2006 (14%). Eight out of 112 PFGE types accounted for 54,9% of isolates studied (n=476/867) which were included in six STs or lineages: ST36 (T12/*emm*12/PFGE.AB;PFGE.AP;others) (n=243, mainly susceptible to macrolides); ST28 (T1/*emm*1/PFGE.X;PFGE.CX;others) (n=125, mainly susceptible to macrolides); ST150 (T8.25.Imp19/*emm*75/PFGE.EF;others) (n=55, mainly susceptible to macrolides); ST406 (T3.13.B3264/*emm*3/PFGE.BG) (n=53, all susceptible to macrolides); ST382 (T6/*emm*6/PFGE.AD;others) (n=42, all susceptible to macrolides); and ST38/ST39 (T4/*emm*4; *emm*stMrp6/PFGE.CZ;others) (n=37, all resistant to macrolides). Of the 113 recurrent carriers, 111 were colonized in 2 or 3 sampling periods and two were colonized in four sampling periods. In the majority of the recurrent carriers (n=88, 77.9%), two to four strains were sampled along the study period. The remaining 25 (22.1%) persons were carriers of a same strain. Also, out of the 113 persons, 54 (47.8%) were carriers at least once of *emm*12 strains. Of the 23 participants

of the multicolonization study, four (17.4%) were colonized by strains of different PFGE types and of a same *emm*-type. An example was one individual that carried two *emm*75 strains (PFGE.CY and PFGE.FO types). The *S. pyogenes* carrier state was seasonal and variable with age and DCC attendance. A very heterogeneous population colonized healthy carriers during 2000-2007. However, some lineages/clones either resistant or susceptible to macrolides were identified as putative poor colonizers and others as widely disseminated and persistent overtime. A replacement of *S. pyogenes* strains was frequently found among recurrent carriers and co-colonization of the oropharynx by multiple strains was detected.

Keywords: *Streptococcus pyogenes*, healthy carriers, longitudinal colonization, co-colonization, lineages

INTRODUCTION

Group A streptococci (GAS) (*Streptococcus pyogenes*) is one of the most versatile and common human pathogens, causing a wide spectrum of diseases ranging from uncomplicated but highly prevalent pharyngitis, to extremely severe infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome (Cunningham, 2000). The carrier state may be clinically important because colonization may provide useful information about the prevalent phenotypes and clones within the community, as they may be present among infections (Durmaz *et al.*, 2003; Fazeli *et al.*, 2003; Hoe *et al.*, 2003; Pires *et al.*, 2005). However, reports on the characterization of GAS from colonization isolates are very insufficient and are useful to assess the role of carriers in the dissemination of GAS and to provide information about the global epidemiology of GAS.

In this study, our aims were to evaluate the trends of oropharyngeal colonization by GAS during 2000 to 2007, to assess the clonal structure of sporadic and persistent strains, to examine the long-term asymptomatic oropharyngeal colonization status and if co-colonization of the oropharynx by multiple strains occurs among asymptomatic persons.

MATERIALS AND METHODS

A– Oropharyngeal colonization (2000-2007)

Study population

The collection of oropharyngeal samples from children attending day-care centers (DCCs) (0-6 yrs), children and adolescents attending schools (7-16 yrs), and close contacts (family and school staff from all institutions) was carried out since 2000 to 2007 throughout a surveillance study in Lisbon area, Portugal. In this study, exclusion criteria for healthy individuals consisted of infection of the upper respiratory tract, febrile disease or antimicrobial usage one-week prior to sampling, and symptoms as fever, throat inflammation and common cold. Other clinical data were obtained prior to sampling through questionnaires given to parents or guardians of all participants to obtain information on recent and past antibiotic usage and associated diseases.

Statistical analysis

Chi-squared test was used to test significance of the observed differences in the standardized frequency of colonization by age group along the study period.

Bacterial identification was carried out by standard methods (Pires *et al.*, 2009). A total of 1,026 GAS were studied, collected from 10,578 throat swab cultures of healthy carriers (9.7%) attending 15 DCCs and five schools during 2000 to 2007: 6,965 throat swabs were from children (0-6 yrs), 2,337 from school-aged children (7-16 yrs) and 1,276 from adults (1,169 from DCC and school staff and 107 from family members).

Macrolide susceptibility testing

All isolates were tested for susceptibility to macrolides (erythromycin, azithromycin, clarithromycin) and lincosamides (clindamycin) by disk diffusion (Oxoid Ltd., Basingstoke, UK), according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2008). The MICs to erythromycin and clindamycin were evaluated by E-test (AB Biodisk®, Solna, Sweden), according to the manufacturer's instructions.

Pulsed-field gel electrophoresis (PFGE)

Macrolide-resistant isolates (n=164) and a subset of macrolide-susceptible isolates (n=703) were characterized according to *Sma*I or *Cfr*9I restriction profiles. Chromosomal DNA preparation and DNA digestion with *Sma*I (New England Biolabs®, Beverly, USA) were performed as previously described (Pires *et al.*, 2009). All M phenotype isolates were analysed

by *Cfr*9I-restriction patterns (*Cfr*9I was from Fermentas, Vilnius, Lithuania), a *Sma*I isoschizomer, since these strains were shown to be uncut by *Sma*I (Cresti *et al.,* 2002; Silva-Costa *et al.*, 2006). The PFGE was run in a CHEF-DRIII apparatus (BioRad, Hercules, USA). The analysis of chromosomal DNA band patterns was performed by visual inspection (Tenover *et al.*, 1995), being a single band difference used as a criterion to define a different pattern, since some patterns presented less than ten bands.

T-typing

Serotyping was carried out by slide agglutination using 5 polyvalent and 21 monovalent anti-T-agglutination sera (Sevapharma, Czech Republic), as described (Pires *et al.*, 2005).

***emm*-typing**

emm gene PCR amplification and assignment of *emm* gene sequence types were carried out as described (www.cdc.gov/ncidod/biotech/strep/protocols.htm).

Multilocus sequence typing (MLST)

PCR amplification of internal fragments of the seven housekeeping genes and assignment of sequence types (ST) were performed as described (http://spyogenes.mlst.net).

B– Longitudinal colonization (2000-2004) and multicolonization (2003)

Study population

The collection of oropharyngeal samples from children attending DCCs (0-6 yrs), children and adolescents attending schools (7-16 yrs), and close contacts (family and school staff from all institutions) was carried out since 2000 to 2004 throughout a surveillance study in Lisbon area, Portugal. The eight participating DCCs and five schools were chosen in collaboration with the School Health and Public Health teams of Centro de Saúde de Oeiras, Oeiras. In this study, exclusion criteria for healthy individuals consisted of infection of the upper respiratory tract, febrile disease or antimicrobial usage one-week prior to sampling, and symptoms as fever, throat inflammation and common cold. Other clinical data were obtained prior to sampling through questionnaires given to parents or guardians of all participants to obtain information on recent and past antibiotic usage and associate diseases.

Sampling

During 12 sampling periods (October, February and May of each school year), a total of 7,894 oropharyngeal samples were taken from 3,900 healthy individuals. For the detection of recurrent carriers - defined as colonized by *S. pyogenes* more than once during two to 12

sampling periods - a total of 1,729 healthy individuals who were sampled more than once were considered for the study (5,723 oropharyngeal samples).

Bacterial identification was carried out by standard methods (Pires *et al.*, 2009). The total number of *S. pyogenes* isolates was 772 (carriage rate 9.8%). A total of 625 isolates were recovered from the 1,729 individuals who were sampled more than once, out of which 243 (38.9%) were from 113 (6.5%) recurrent carriers. These isolates were considered as associated with long-term or longitudinal colonization. During May, 2003, all beta-hemolytic colonies identified as *S. pyogenes* that were picked from each of the primary plates containing the swabs of 23 participants (2.7%) out of 865 persons sampled were considered as causing co-colonization or multicolonization of the same niche.

Molecular typing

Isolates of both groups - 243 causing longitudinal colonization and 74 isolates causing multicolonizaton - were typed by PFGE (Pires *et al.*, 2009) for strain definition and by sequencing part of the *emm* gene encoding the M surface protein as described by the *Streptococcus* laboratory from the Centers for Disease Control and Prevention (http:/www.cdc.gov/ncidod/biotech/strep/protocols.htm).

RESULTS

A– Oropharyngeal colonization (2000-2007)

Frequency of *S. pyogenes* oropharyngeal colonization

According to the age group, variable frequencies of oropharyngeal colonization were observed (Fig. 2.1). The mean carrier rate of the children aged 0-6 years was higher (11.6%; $p<0.05$) than of the children aged 7-16 years (7.8%; $p<0.05$). GAS carriers aged 0-16 years were 10.6% (n=990/9,302). Among adults, children's family members exhibited a higher frequency of *S. pyogenes* colonization (8.4%, $p<0.05$) when compared with DCC/school staff (2.6%, $p>0.05$).

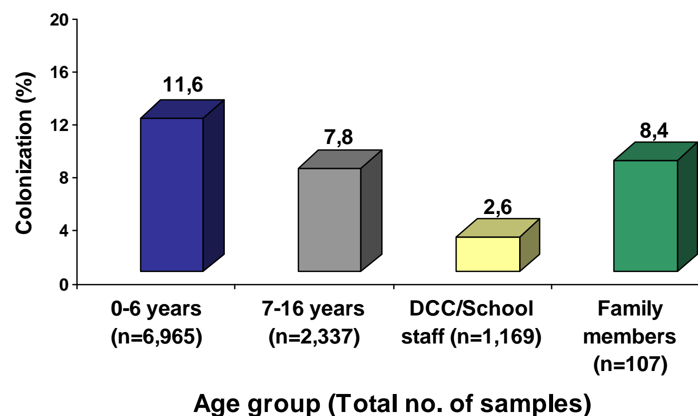

Figure 2.1. Frequency of *S. pyogenes* oropharyngeal colonization by age group of the surveyed individuals.

According to the school period, peaks of high colonization rates (>10%) were observed during the three periods (Fig. 2.2): Winter (February) of 2001-2003 and 2007, Spring (May) of 2001 and 2004, and Autumn (October) of 2001-2003. The frequency of colonization in February was higher when compared with October and May (2000 to 2004), except in the 2003/2004 period, when the higher value of colonization was observed in May (18%), compared with February (8%).

We also observed variable frequencies of colonization with institution and overtime. Among the five DCCs participating along the study period (n=5, codes 1, 3, 5, 7, 8), colonization varied from a minimum of 0% to a maximum of 49% (Fig. 2.3) whereas among the schools (n=5, codes A, B, C, D, E), colonization rates were between 4% and 16% (Fig 2.4).

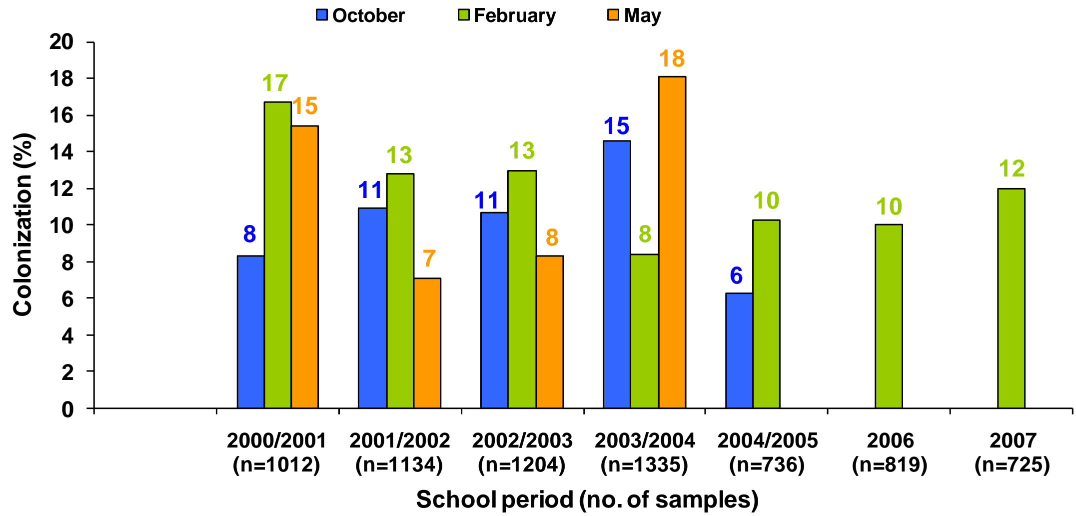

Figure 2.2. Frequency of *S. pyogenes* oropharyngeal colonization by school period.

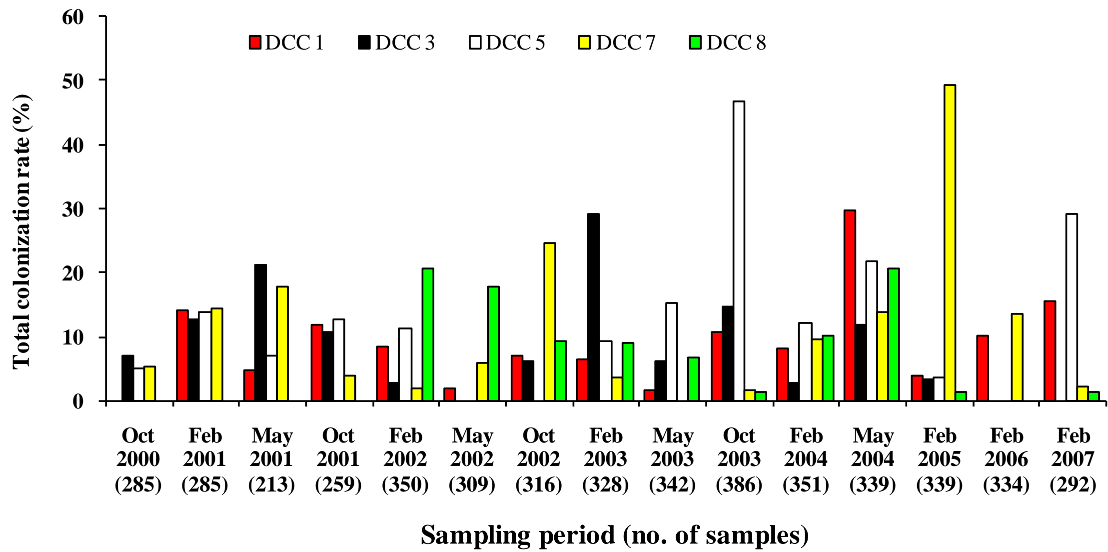

Figure 2.3. Variation of colonization rates among DCCs that participated along the study period (2000-2007).

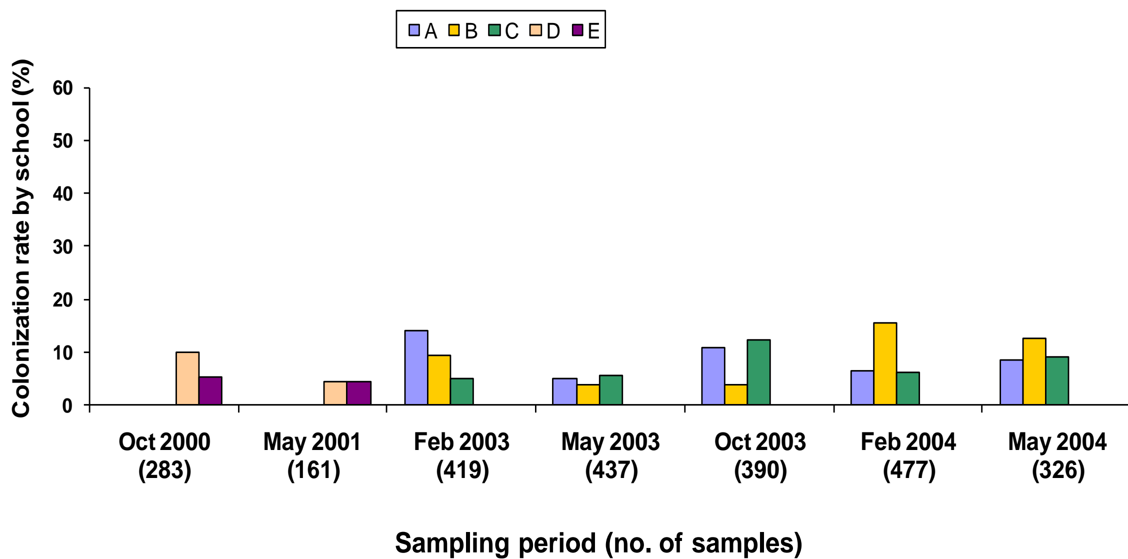

Figure 2.4. Variation of colonization rates among schools.

Resistance to macrolides and clindamycin

Globally, resistance to macrolides was 16.0% (n=164/1,026) and to clindamycin was 4.2% (n=43/1,026). Resistance to macrolides was stable (approximately 10%) during 2000-2002 and reached the higher value (28%) in 2003 (Fig. 2.5); then, a decrease was observed in 2004-2005 (20% and 3%, respectively); in 2006 the resistance rate increased again (14%), and in 2007 presented a slight decrease (10%). Resistance to clindamycin was lower than 10% among all years, except in 2006 (14%).

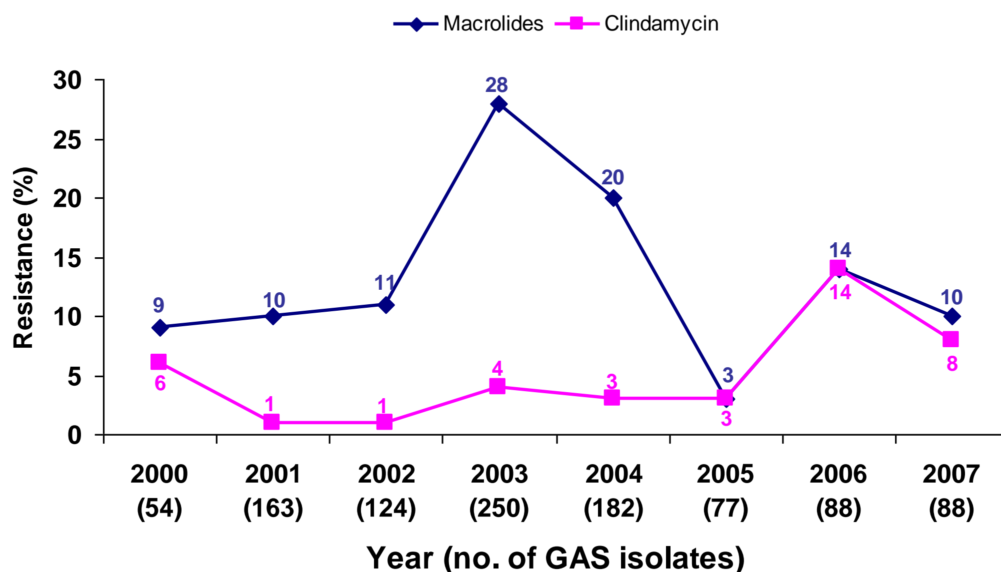

Figure 2.5. Resistance to macrolides and clindamycin along the study period (2000-2007).

Clonality and major lineages

A total of 112 different *Sma*I/*Cfr*9I PFGE patterns were found among 867 isolates tested and 57 of them included single isolates. Eight major patterns included 54,9% (n=476/867) of the isolates and were grouped into six lineages (Fig. 2.6): PFGE.AB/PFGE.AP/others (n=243, mainly macrolide-susceptible isolates) – mainly T12, *emm*12, ST36; PFGE.X/PFGE.CX/others (n=125, mainly macrolide-susceptible isolates) – mainly T1, *emm*1, ST28 ; PFGE.EF/others (n=55, mainly macrolide-susceptible isolates) – mainly T8.25.Imp19, *emm*75, ST150; PFGE.BG (n=53 macrolide-susceptible isolates) – mainly T3.13.B3264, *emm*3, ST406 ; PFGE.AD/others (n=42 macrolide-susceptible isolates) – mainly T6, *emm*6, ST382; and PFGE.CZ/others (n=37 macrolide-resistant isolates) – T4, *emm*4/*emm*stMrp6, ST39/ST38. The replacement and fluctuation of the six major lineages were observed along the study period. Lineage ST28 prevailed in 2000 with a frequency of 10.2% (5 isolates), was replaced by lineage ST406 with a prevalence of 23.8% (34 isolates) in 2001, which was also replaced by lineage ST36 with a major frequency of 38.3% and 36.4% (44 and 87 isolates, respectively) in 2002 and 2003. In 2004, lineages ST28 and ST36 were dominant with prevalences of 26.5% and 25.9% (43 and 42 isolates, respectively), in 2005 ST36 was the major lineage (45.6%, 26 isolates) and, in 2006 ST150 and ST36 prevailed with frequencies of 29.2% and 26.2% (19 and 17 isolates, respectively). Finally, in 2007 ST36 (PFGE.AB; PFGE.AP) was dominant again with prevalence of 48.6% (18 isolates).

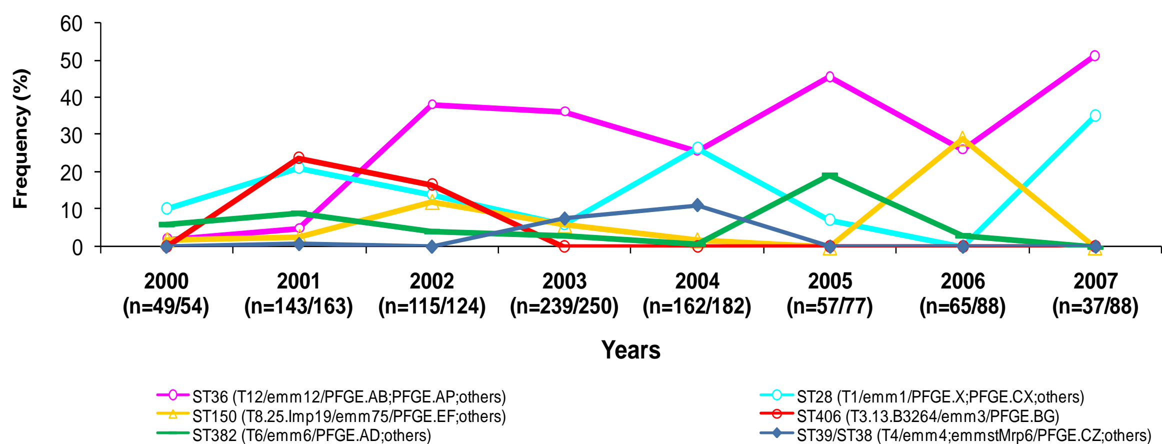

Figure 2.6. Evolution of frequencies of the six major clonal lineages from oropharyngeal colonization.

Minor lineages

Out of ten minor lineages, four were predominant during the study period (Fig. 2.7): ST55 (mainly T2.28, *emm*2, PFGE.BH/others) in 2000 (n=15 macrolide-susceptible isolates); ST15 (mainly T3.13.B3264, *emm*3, PFGE.BM/other) in 2001 (n=13 macrolide-susceptible isolates); ST52 (mainly T28, *emm*28, PFGE.F/PFGE.AK/others) in 2006 (n=12 macrolide-resistant

isolates); and ST408 (mainly T3.13.B3264, *emm*3, PFGE.AJ) in 2006 (n=12 macrolide-susceptible isolates).

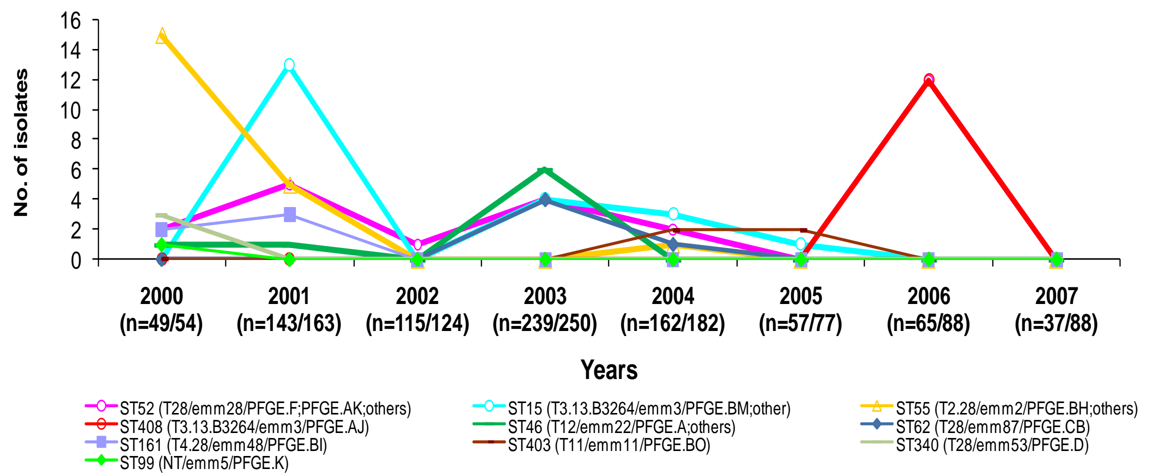

Figure 2.7. Evolution of frequencies of the ten minor clonal lineages from oropharyngeal colonization.

GenBank accession number

The sequence of the new *emm*-type st4050.0 was submitted to GenBank database under the accession number FJ711065.

B– Longitudinal colonization (2000-2004) and multicolonization (2003)

Longitudinal colonization

The mean number of samples per individual was 2.0 (7,894/3,900), ranging for a minimum of 1 to a maximum of 12. Of the 113 recurrent carriers, 98, 13 and two individuals were colonized in two, three and four sampling periods, respectively. By age group, a total of 83 (73.4%) individuals were distributed in the group 0 to six years-old, 29 (25.7%) among seven to 16 years-old and one individual (0.9%) was an adult.

emm-types and PFGE patterns

A total of 20 *emm*-types were detected in isolates from recurrent carriers, and seven included the vast majority of isolates (n=201, 82.7%): *emm*12 (n=72, 29.6%), *emm*1 (n=37, 15.2%), *emm*4 (n=23, 9.5%), *emm*3 (n=21, 8.6%), *emm*89 (n=18, 7.4%), *emm*2 (n=16, 6.6%) and *emm*75 (n=14, 5.8%). Other *emm*-types found in this study were: *emm*6 (n=8), *emm*77 (n=7), *emm*48 (n=6), *emm*11 (n=4), *emm*87 (n=4), *emm*29 (n=3), *emm*28 (n=2), *emm*58 (n=2), *emm*stMrp6 (n=2), *emm*44 (n=1), *emm*78 (n=1), *emm*88 (n=1) and *emm*102 (n=1).

A total of 48 PFGE patterns were identified and six accounted for the majority of isolates (n=128, 53%) and were usually associated with one *emm* type (Fig. 2.8). The PFGE types most

found among the 113 recurrent carriers were: PFGE.AB (n=31 carriers, 27.4%), PFGE.X (n=26, 23.0%) and PFGE.AP (n=23, 20.4%).

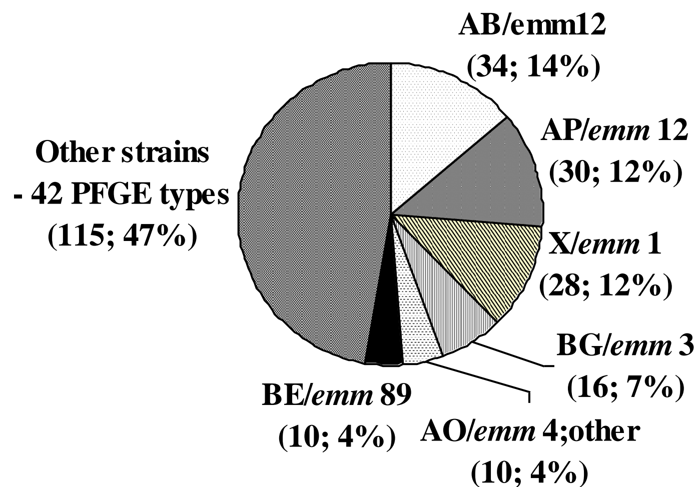

Figure 2.8. Distribution of PFGE types and associated *emm* types among isolates from recurrent carriers.

Patterns of streptococcal colonization

Among most of the recurrent carriers (n=88, 77.9%), two to four strains were isolated along the study period; strain replacement occurred among 49 of those recurrent carriers (55.7%): common replacements were PFGE.AB/*emm*12 by PFGE.BG/*emm*3 and PFGE.CN/*emm*89 by PFGE.X/*emm*1 (n=3 carriers, each). The remaining 25 (22.1%) persons were carriers without strain replacement. Also, out of the 113 persons, 54 (47.8%) were carriers at least once of *emm*12 strains.

Multicolonization

Of the 23 participants of the multicolonization study, only four (17.4%) were colonized by multiple strains of different PFGE types and of a same *emm* type: individual XIV carried two *emm*12 strains (PFGE.AP and PFGE.FQ types); individual XV carried two *emm*22 strains (PFGE.A and PFGE.B); individual XVI carried two *emm*75 strains (PFGE.FO and PFGE.CY); and individual XX carried two *emm*9 strains (PFGE.FB and PFGE.FM) (Table 2.1).

Table 2.1. Characteristics of *S. pyogenes* isolates recovered from individuals included in the multicolonization study.

Individual	*S. pyogenes* isolates	
	Total number	PFGE/*emm*-types
I	3	PFGE.BE/*emm*89 (n=3)
II	3	PFGE.BE/*emm*89 (n=3)
III	4	PFGE.BE/*emm*89 (n=4)
IV	4	PFGE.AP/*emm*12 (n=4)
V	2	PFGE.AP/*emm*12 (n=2)
VI	2	PFGE.AP/*emm*12 (n=2)
VII	3	PFGE.FP/*emm*11 (n=3)
VIII	5	PFGE.BK/*emm*12 (n=5)
IX	2	PFGE.BK/*emm*12 (n=2)
X	3	PFGE.AB/*emm*12 (n=3)
XI	3	PFGE.FE/*emm*11 (n=3)
XII	2	PFGE.AO/*emm*4 (n=2)
XIII	3	PFGE.FE/*emm*11 (n=3)
XIV	4	PFGE.AP/*emm*12 (n=3)
		PFGE.FQ/*emm*12 (n=1)
XV	3	PFGE.B/*emm*22 (n=2)
		PFGE.A/*emm*22 (n=1)
XVI	5	PFGE.FO/*emm*75 (n=3)
		PFGE.CY/*emm*75 (n=2)
XVII	5	PFGE.AO/*emm*4 (n=5)
XVIII	4	PFGE.F/*emm*28 (n=4)
XIX	3	PFGE.AW/*emm*42 (n=3)
XX	2	PFGE.FB/*emm*9 (n=1)
		PFGE.FM/*emm*9 (n=1)
XXI	3	PFGE.AL/*emm*9 (n=3)
XXII	2	PFGE.CY/*emm*75 (n=2)
XXIII	4	PFGE.DM/*emm*12 (n=4)

DISCUSSION

A– Oropharyngeal colonization (2000-2007)

The asymptomatic carrier state presents some important aspects. Carriers should infect other individuals from close contacts. However, the frequency of bacterial transmission seems to be lower when compared with an infected individual with manifestations of symptoms (Pichichero and Casey, 2003). On the other hand, studies performed by Pichichero and Casey (2003) showed that the incidence of clinical disease among individuals infected by one carrier of *S. pyogenes* could vary between 4% and 89%. So, the study of carriers could help to understand the global epidemiology of this pathogen and the factors (of both host and bacteria) that control the development of disease, as well as its degree of severity.

The *S. pyogenes* carrier state was seasonal and variable with age and DCC attendance. The global prevalence of healthy *S. pyogenes* carriers aged 0-16 years was 10.6%, which was higher than some previous findings (2.5-6.8%) (Pichichero *et al.*, 1999; González-Lama *et al.*, 2000; Herruzo *et al.*, 2002), although smaller than others (14.3-32%) (Durmaz *et al.*, 2003; Martin *et al.*, 2004). The prevalence of *S. pyogenes* in healthy individuals was described as being low before the age of three years (1.9%-7.1%) and in adults ≥16 years (2.4%-3.7%) and highest in the age group 3-15 years (5.0%-21.2%) (Gunnarsson *et al.*, 1997). It is also documented that the peak of incidence of infections caused by *S. pyogenes* occurs among school children (5-15 years) (Cunningham, 2000; Martin *et al.*, 2004). In the present study, the mean carrier rate among pre-school children (0-6 years) was higher (11.5%) than among school-aged children (7-16 years), although both mean frequencies were included in the range published by Gunnarsson and colleagues (1997) for the age group 3-15 years. The crowding of children in day-care centers may possibly increase the carrier rate in healthy pre-school children and a mechanism whereby children gradually acquire immunity to the prevalent serologic types of GAS has been documented (Quinn, 1980). The results described in the present study partially confirmed what was reported by these authors; however it was observed a higher colonization frequency among pre-school children than among school children. These results reinforce the notion that the exposition to this pathogen along the years leads to the production of antibodies, becoming the individuals gradually immune to *S. pyogenes*. Therefore, continuous epidemiological surveillance in the community is crucial to assess the role of carriers as potential transmission vectors of specific strains associated with streptococcal disease. Besides, carriage isolates can be used as controls as they do not cause disease.

A total of 164 *S. pyogenes* macrolide-resistant isolates were identified during 2000 to 2007 among the 1,026 studied isolates (16.0%). This rate, even being high, was lower when compared with those from clinical *S. pyogenes* isolates from various origins (strains associated

with invasive and noninvasive disease) documented in Portugal: 35.8% in 1998-1999 (Melo-Cristino and Fernandes, 1999), 26.6% between 1998 and 2003 (Silva-Costa *et al.*, 2005) and 27.4% in 1999-2002 (Pires *et al.*, 2005). The PROTEKT multicentric study reported 24% of macrolide resistance among clinical isolates from respiratory infections in Portugal (Cantón *et al.*, 2002). This latter study also reported high frequencies of macrolide resistance among isolates from Poland, Hong-Kong, Italy and Spain, in contrast with Indonesia, Austria, Belgium, Netherlands and United Kingdom, where resistance to macrolides was absent. Resistance to macrolides in the United States has been variable, ranging high values in specific populations (48%) (Hasenbein *et al.*, 2004). In a study from 2000 to 2001 (Green *et al.*, 2004), a low level of resistance during the first seven months of the study was observed (3.7%), contrasting with the last two months, when resistance increased to 35%. It is important to notice that the values published by these authors are related with *S. pyogenes* isolates from individuals presenting symptoms of respiratory infection. Resistance to macrolides in *S. pyogenes* from asymptomatic carriers was documented as variable in other countries: in 2001-2002 was 32.9% in Republic of Korea (Kim and Uh, 2004), a value higher than the reported in the present study; in contrast, between 1995 and 2001 in Bulgaria, it was detected a frequency of macrolides resistance of 2.1% (Detcheva *et al.*, 2002) among isolates from colonization but also among symptomatic individuals, and in Turkey, no macrolides resistance was detected (Durmaz *et al.*, 2003). The values reported by these two authors are very lower compared with those described in our study. Several factors can be related with the different macrolides resistance rates in *S. pyogenes* from different countries. One could be the cross-transmission of resistant strains and other could be the horizontal transfer of resistance genes from resistant to susceptible strains. The selective pressure caused by the inappropriate use of antibiotics (e.g. macrolides) seems to be the most important factor, could helping the dissemination of resistance determinants among *S. pyogenes* population.

A very heterogeneous population of *S. pyogenes* colonized healthy carriers. However, some lineages/clones either resistant or susceptible to macrolides were identified as widely disseminated and persistent overtime: ST28, ST36, ST38/39, ST150, ST382 and ST406. ST382 and ST406 were detected before in several European countries, whereas ST28, ST36, ST38/39 and ST150 were found in several European countries and also in American and Asian countries, associated both with noninvasive or invasive disease and, as reported here, also associated with asymptomatic oropharyngeal carriage in Portugal (http://spyogenes.mlst.net, last search at September 15, 2011). These observations confirm that the same clones that can cause noninvasive or invasive disease may also include isolates colonizing the throat of individuals without any symptoms of clinical illness. Hence, it is important to understand the specific strain, host, and environmental factors that contribute to the prevalence of a *S. pyogenes* clone in pharyngitis cases, invasive disease or asymptomatic carriers. It is tempting to assume that host

factors may play an important role in the development of disease, and in fact the role of host genetic factors in determining susceptibility to streptococcal infections has been studied (Kotb *et al.*, 2003).

B– Longitudinal colonization (2000-2004) and multicolonization (2003)

To the best of our knowledge, this study represents the largest longitudinal study carried out in several DCCs and schools that has used PFGE and *emm*-typing techniques to characterize all *S. pyogenes* isolates.

In previous longitudinal studies, carriage rates were reported to be extremely high (up to 25%) (Quinn and Federspiel, 1973; Quinn, 1980), which are in contrast with the rate found in our study (9.8%). We found that a high diversity of *S. pyogenes* strains was associated with long-term colonization: they were from 48 PFGE patterns and from 20 *emm*-types. This diversity of *emm*-types was higher than the observed in other four-year longitudinal study (Martin *et al.*, 2004), where 13 different *emm*-types circulated in that period.

A replacement of *S. pyogenes* strains was frequently found among recurrent carriers and co-colonization of the oropharynx by multiple *S. pyogenes* strains was detected. *emm*12 isolates seem to be successful colonizers as they were identified as widely disseminated among carriers and were persistent overtime. It may have clinical relevance since association between *emm*12 and *S. pyogenes* pharyngitis or paediatric invasive *S. pyogenes* infections has been reported in Portugal (Friães *et al.*, 2007; Silva-Costa *et al.*, 2008)

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All the experimental work was performed by R. Pires, with the exception of the identification and characterization of part of the isolates which was carried out with the collaboration of Paula Fernandes and other graduate and undergraduate students of the laboratory.

Chapter III

DESCRIPTION OF MACROLIDE-RESISTANT AND POTENTIAL VIRULENT CLONES OF *STREPTOCOCCUS PYOGENES* CAUSING ASYMPTOMATIC COLONIZATION DURING 2000-2006 IN LISBON AREA

These results were published in:
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ABSTRACT

The asymptomatic oropharyngeal colonization rate by *Streptococcus pyogenes* was 10.7% in children (901 among 8,405 children 0-16 years-old) and 3,3% in adults (37 among 1,126 households of children) in the Lisbon area during 2000-2006. Macrolide-resistant *S. pyogenes* from children (n=149) was variable with time: 9.8%-10.7% in 2000-2002, 28.1% in 2003, 19.6%-2.7% in 2004-2005 and 14.6% in 2006. Eight lineages (97.3% of isolates) were identified based on at least 80% similarity of PFGE patterns, T-types, *emm*-types and multilocus sequence types (ST). The elevated frequency of macrolide resistance was associated with M phenotype lineages I (*emm*12/ST36) and V (*emm*4, *emm*75/ST39 and a novel *emm*stMrp6 type) and with one $cMLS_B$ lineage IV (*emm*28/ST52) known as associated with upper respiratory tract and invasive infections. Significant associations ($p<0.05$) between *emm* type/virulence genotype were found, such as *emm*1/$speA^+ssa^-$, *emm*4/ssa^+prtF1^+, *emm*12/$speA^-ssa^-$. The high prevalence (>20%) of *speC*, *prtF1* or *ssa* was probably caused either by clonal dissemination (*speC*), or to horizontal gene transfer events (*prtF1* and *ssa*). This report contributes to a better understanding of the molecular epidemiology and evolution of macrolide-resistant *S. pyogenes* causing symptom-free oropharyngeal colonization. These colonizing strains carry macrolide resistance and virulence genes capable of being transferred to other bacterial species sharing the same niche.

Keywords: *Streptococcus pyogenes*, colonization, macrolides, resistance, virulence, lineages.

INTRODUCTION

Streptococcus pyogenes (or Group A *Streptococcus* - GAS) is strictly a human pathogen, which is able to cause several disease manifestations, including mild infections, like pharyngitis or impetigo, severe systemic diseases, such as necrotizing fasciitis or streptococcal toxic shock syndrome (STSS), as well as post infection complications (Cunningham, 2000). Asymptomatic carriers of *S. pyogenes* harbour the organism in the nose or throat and display no symptoms of acute infection (Pichichero *et al.*, 1999). Because the carriage state can persist for months, the organism remains capable of transmission to a new host, what makes crowded institutions, like day-care centers, propitious for its spread (Fazeli *et al.*, 2003; Aguero *et al.*, 2008).

S. pyogenes is uniformly susceptible to penicillin and this is the universal recommended treatment for *S. pyogenes* infections. However, macrolides represent an alternative in patients who are allergic to penicillin (Bisno *et al.*, 2002), and consequently increased frequency of macrolide-resistant *S. pyogenes* from infection sites has been reported in different countries (Felmingham *et al.*, 2004) including from asymptomatic oropharyngeal colonization (Creti *et al.*, 2005; Pires *et al.*, 2005; Chang *et al.*, 2010).

Macrolide resistance in *S. pyogenes* can be caused by an efflux system that is encoded by the *mef*(A) gene. This mechanism is responsible for cross resistance to 14- and 15-membered macrolides, but does not affect the activity of 16-membered macrolides and lincosamides (Sutcliffe *et al.*, 1996). Another mechanism, which involves the erythromycin ribosome methylation (*erm* genes), leads to resistance to all classes of macrolides, lincosamides and streptogramins B (Seppälä *et al.*, 1998). This may have impact on treatment of severe diseases where clindamycin has a major role.

The variety of diseases caused by GAS is most probably due to the diversity of virulence gene products (Cunningham, 2000) mainly encoded by mobile genetic elements, such as prophages. Streptococcal pyrogenic exotoxins (SpeA or SpeC) and streptococcal superantigen (Ssa) are bacteriophage-encoded virulence factors and are associated with the development of invasive diseases (McCormick *et al.*, 2000), whereas streptococcal invasin PrtF1 mediates adherence and subsequent internalization to host epithelial cells (Talay *et al.*, 1992).

This work aimed to describe the frequency of macrolide-resistant *S. pyogenes* recovered in Lisbon area, Portugal, from healthy carriers during 2000 to 2006 and to assess the capacity of asymptomatic carriers as reservoirs of macrolide-resistant and potential virulent clones capable of causing a wide spectrum of infections.

MATERIALS AND METHODS

Study population

A total of 9,531 oropharyngeal samples were taken from children attending day-care centers (DCCs) (0-6 yrs) (n=6,240), children and adolescents attending schools (7-16 yrs) (n=2,165), and close contacts (family and school staff from all institutions) (n=1,126), during a surveillance study in the Lisbon area from 2000 to 2006 that aimed to evaluate the colonization rates along the time, the antimicrobial resistance patterns and the clonal structure of the isolates. Fifteen DCCs and five schools were enrolled in the study and were chosen in collaboration with the School Health and Public Health teams of a Primary Care Centre (Centro de Saúde de Oeiras, Oeiras - CSO). Authorization for collecting the samples was obtained by the health teams of CSO. Informed consent was obtained prior to sampling given by the parents or guardians of the children. Demographic, family data and clinical history such as past episodes of tonsillitis, pharyngitis and scarlet fever, recent and past antibiotic usage of both the children and household members were obtained prior to sampling through questionnaires filled by parents or guardians of children and by the adult participants. Participants were volunteers and sampling was random. In this study design, exclusion criteria for healthy individuals consisted of presentation of clinical manifestations, such as fever, throat inflammation or common cold, and associate diseases.

Identification of the *S. pyogenes* isolates

A total of 938 (9.8%) single isolates were collected: 901 were from children and 37 were from adults (colonization rates of 10.7% and 3.3%, respectively). Isolates were identified by standards methods, as previously described (Pires *et al.*, 2009).

Susceptibility testing and macrolide resistance phenotypes

Susceptibility to erythromycin, clarithromycin, azithromycin and clindamycin was tested by disk diffusion (Oxoid, Basingstoke, UK), according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2006) and the macrolide resistance phenotypes were determined by a double-disk test, as previously described (Seppälä *et al.*, 1993).

Minimum inhibitory concentrations (MIC)

MIC to erythromycin and clindamycin were performed to all macrolide-resistant isolates by E-Test (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions.

Pulsed-field gel electrophoresis (PFGE) and dendrogram construction

Chromosomal DNA preparation and digestion with *Sma*I (New England Biolabs®, Beverly, USA) or *Cfr*9I (Fermentas, Vilnius, Lithuania) were performed as previously described (Pires *et al.*, 2009). The analysis of chromosomal DNA band patterns was first performed by visual inspection, and since the number of DNA fragments was frequently less than 10, a single band difference was used as a criterion to define a different pattern, as suggested (Tenover *et al.*, 1995). The relationship among all patterns was assessed in a dendrogram generated by the software package BioNumerics® version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium): resemblance was computed with Dice coefficient and agglomerative clustering was performed with the unweighted pair-group method with arithmetic mean (UPGMA), with optimization and tolerance values of 1% and 1.5%, respectively.

Polymerase chain reaction (PCR)

Template DNAs were isolated as described (Klugman *et al.*, 1998). **a) Detection of macrolide resistance genes.** All macrolide-resistant isolates were analysed for the presence of *mef*(A), *erm*(A) and *erm*(B) genes, as previously described (Figueira-Coelho *et al.*, 2004). **b) Detection of virulence genes.** All macrolide-resistant isolates were analysed for detection of *speA* and *speC* genes, encoding streptococcal pyrogenic exotoxins, the *ssa* gene, encoding a superantigen, and the *prtF1* gene, encoding a fibronectin-binding protein (Pires *et al.*, 2009).

T-typing

All macrolide-resistant isolates were analysed for assignment of T capsular serotypes (T-types). The method was performed by slide agglutination using 5-polyvalent and 21-monovalent anti-T-agglutination sera (Sevapharma, Prague, The Czech Republic).

Sequence-based typing methods

a) *emm*-typing. All macrolide-resistant isolates were analyzed for assignment of *emm* types as described by the *Streptococcus* laboratory from the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). **b) Multilocus sequence typing (MLST).** Representative isolates of major clonal groups (with more than three isolates) were selected for assignment of sequence types (ST) according to the *S. pyogenes* MLST method and database (http://spyogenes.mlst.net/).

Statistical analysis

The χ^2 test was used when appropriate. Differences were considered significant at $p<0.05$.

RESULTS

Total and annual macrolide resistance rates

The frequency of macrolide-resistant isolates among all the populations during the seven-year study was 16.5% (155/938). Macrolide resistance was higher among 0-6 years-old children (n=136/747, 18.2%) and among adults (n=6/37, 16.2%) comparing with 7-16 years-old children (n=13/154, 8.4%) ($p<0.05$). The frequency of macrolide resistance among children changed along the study period: it was stable during 2000-2002 (9.3% to 11.3%), reached the higher value during 2003 (28.0%), decreased in 2004 and 2005 (19.8% and 2.6%, respectively) and increased in 2006 (13.6%) (Fig. 3.1).

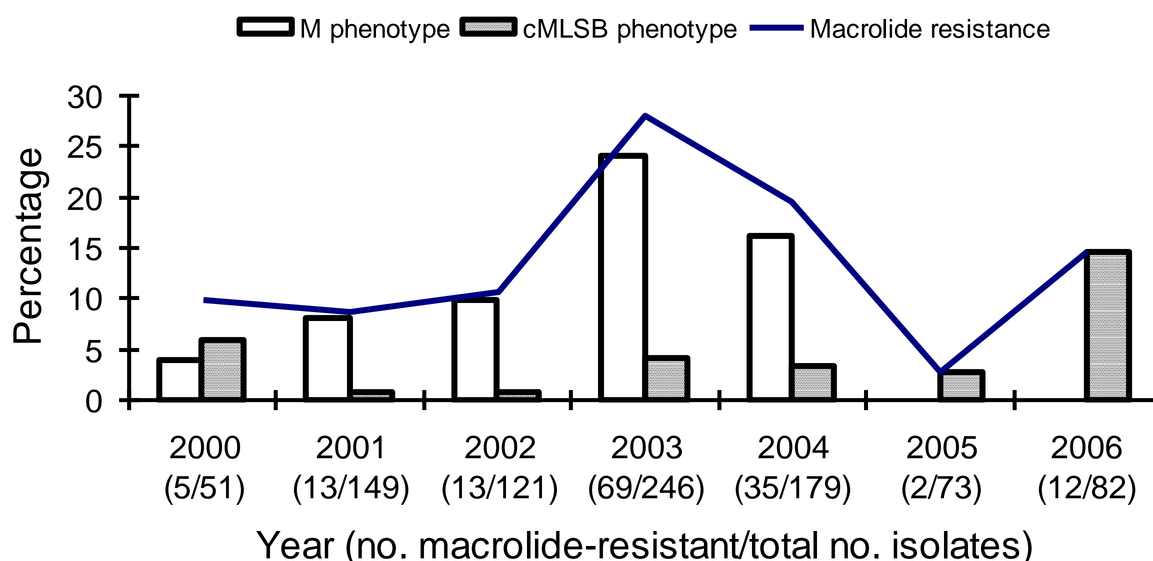

Figure 3.1. Temporal evolution of macrolide resistance frequency and phenotypes among *S. pyogenes* colonization isolates in children from Lisbon area (2000-2006).

Legend: M phenotype – resistance to 14- and 15-membered macrolides and susceptibility to lincosamides and streptogramin B; $cMLS_B$ phenotype – resistance to macrolides, lincosamides and streptogramin B.

Usage of antimicrobials by surveyed individuals

Out of the 901 positive throat swabs of children, six (0.7%) were obtained from individuals who were taking antimicrobial(s) at the time of sampling (Table 3.1), but only one individual reported consumption of macrolide (clarithromycin); out of these six positive samples, one macrolide-resistant isolate was recovered, and it was from the individual who undertook the clarithromycin therapy. The information regarding antimicrobial consumption prior to sampling is included in Table 3.1.

Macrolide resistance phenotypes and genotypes

Totally, among the 149 macrolide-resistant isolates from children, 76.5% were of M phenotype (n=114) and 23.5% were of the constitutive MLS_B phenotype ($cMLS_B$) (n=35). The M phenotype isolates were low-level resistant to erythromycin (MIC 1-18 µg/mL), susceptible to clindamycin (MIC 0.016-0.125 µg/mL), and only carried the *mef*(A) gene. The $cMLS_B$ phenotype isolates were high-level resistant to erythromycin and clindamycin (MIC>256 µg/mL) and only carried the *erm*(B) gene. None of macrolide-resistant isolates carried the *erm*(A) gene. The distribution of both macrolide-resistant phenotypes was variable along the years (Fig. 3.1). The M phenotype isolates increased from 3.9% in 2000 to 24.0% in 2003 and were not found during years 2005-2006. The increased prevalence of the M phenotype in 2003-2004 was statistically significant ($p<0.05$) when compared with the prevalence observed in 2000-2002. The $cMLS_B$ phenotype isolates were prevalent in 2000 and later in 2005-2006. The increased frequency of the $cMLS_B$ phenotype in 2006 was statistically significant ($p<0.05$) when compared with the frequencies observed prior 2005.

Virulence genes

The frequency of virulence genes under study among the children isolates was not similar: the *prtF1* gene was the most frequent one (43%), followed by *ssa* (38%), *speC* (23%) and *speA* (13%). Six *emm* types were associated with particular virulence profiles in a majority of isolates ($p<0.05$) (Table 3.2): *emm*12 ($speA^-$, ssa^-, $prtF1^-$), *emm*4 ($speA^-$, ssa^+, $prtF1^+$), *emm*28 (ssa^-, $prtF1^-$), *emm*75 ($speC^-$, ssa^+, $prtF1^+$), *emm*1 ($speA^+$, $speC^-$, ssa^-, $prtF1^-$) and *emm*22 ($speC^+$, ssa^+, $prtF1^+$).

Clonal lineages

The dendrogram of all the *Cfr*9I and *Sma*I patterns of the M and $cMLS_B$ phenotype children isolates is shown in Figure 3.2. Considering a cut-off of 80% similarity, eight PFGE clonal groups or lineages were identified, which included 97.3% of the isolates (n=145). The remaining isolates (n=4) were included in minor lineages. Table 3.3 shows the genotypic characteristics of the isolates and their distribution over time. Lineage I included 44 isolates (29.5%) of M phenotype and *emm*12 of which a large proportion were T12 (47.7%, n=21) and nontypeable (50%, n=22). The five isolates of this lineage tested by MLST were ST36. Lineage V included 40 isolates (26.8%) of M phenotype, *emm*4 (95.0%; n=38), *emm*75 (n=1) or *emm*stMrp6 (n=1). The six isolates tested by MLST were ST39 (n=5) and ST38 (n=1), which are single locus variants (SLV). The *emm*stMrp6 type was found in this study (GenBank under

Table 3.1. Antimicrobial consumption among children carriers of macrolide-resistant and -susceptible *S. pyogenes* isolates at sampling period, 30 days and 2-6 months before sampling, during 2000-2006 in Lisbon area, Portugal.

S. pyogenes collection (no. of isolates)	No. of sampled individuals with antibiotherapy								
	At day of sampling			30 days before sampling			2-6 months before sampling		
	Yes (macrolides) [a]	No [b]	NK/NA [c]	Yes (macrolides) [a]	No [b]	NK/NA [c]	Yes (macrolides) [a]	No [b]	NK/NA [c]
Macrolide-resistant (n=149)	1 (1)	139	9	13 (0)	129	7	41 (3)	99	9
Macrolide-susceptible (n=752)	5 (0)	690	57	83 (5)	608	61	209 (11)	461	82
Total (n=901)	6 (1)	829	66	96 (5)	737	68	250 (14)	560	91

Legend: [a] Individuals who took antimicrobials (in brackets, individuals who took macrolides), [b] Individuals who did not take antimicrobials, [c] Individuals who did not know or did not answer to the questionnaire.

Table 3.2. Distribution of virulence genes among *emm* types of macrolide-resistant *S. pyogenes* from oropharyngeal colonization in children (2000-2006).

emm type (no. of tested isolates)	Virulence genes [a]							
	speA		*speC*		*ssa*		*prtF1*	
	pos	neg	pos	neg	pos	neg	pos	neg
12 (45)	**2**	**43**	13	32	**0**	**45**	**9**	**36**
4 (38)	**1**	**37**	6	32	**35**	**3**	**31**	**7**
28 (20)	0	20	6	14	**0**	**20**	**5**	**15**
75 (17)	0	17	**0**	**17**	**13**	**4**	**9**	**8**
1 (14)	**14**	**0**	**0**	**14**	**0**	**14**	**1**	**13**
22 (8)	1	7	**5**	**3**	**7**	**1**	**7**	**1**
11 (4)	0	4	1	3	0	4	1	3
6 (1)	0	1	1	0	0	1	0	1
44 (1)	1	0	1	0	0	1	1	0
stMrp6 (1)	0	1	1	0	1	0	0	1
Total (149)	19	130	34	115	56	93	64	85

Legend: [a] Pos – no. of isolates that presented the virulence gene; Neg – no. of isolates that did not carry the virulence gene; in bold are indicated the statistically significant differences between presence and absence of virulence genes.

accession no. FJ711062). Lineage IV (n=20, 13.4%) was of cMLS$_B$ phenotype, *emm*28, T28 and ST52 (n=5 isolates tested). Lineage VIII (n=14; 9.4%) was of M phenotype, *emm*1, T1 and ST28 (n=2 isolates tested). The remaining four lineages (VII, III, VI and II) were less prevalent (< 10 isolates).

Temporal evolution of macrolide-resistant isolates

In 2000, no prevalent lineages were found (Table 3.3). During 2001-2004, when M phenotype was dominant (see Fig. 3.1), clonal variations were observed. For instance, lineage VIII (n=14 isolates, *emm*1/ST28/M phenotype) was predominant in 2001 (n=11), lineage I (n=44 isolates, *emm*12/ST36/M phenotype) was prevalent in 2002 (n=10) and in 2003 (n=27). In 2003, when macrolide resistance was higher (28.1%) (see Fig. 3.1), lineage V (n=40 isolates, *emm* variable/ST39) was also frequent (n=20) and it remained frequent in 2004 (n=19). In the 2005-2006 period, all macrolide-resistant isolates (n=14) were of the cMLS$_B$ phenotype of lineage II (n=2, *emm*11/ST403) in 2005, and of lineage IV (n=12, *emm*28/ST52) in 2006.

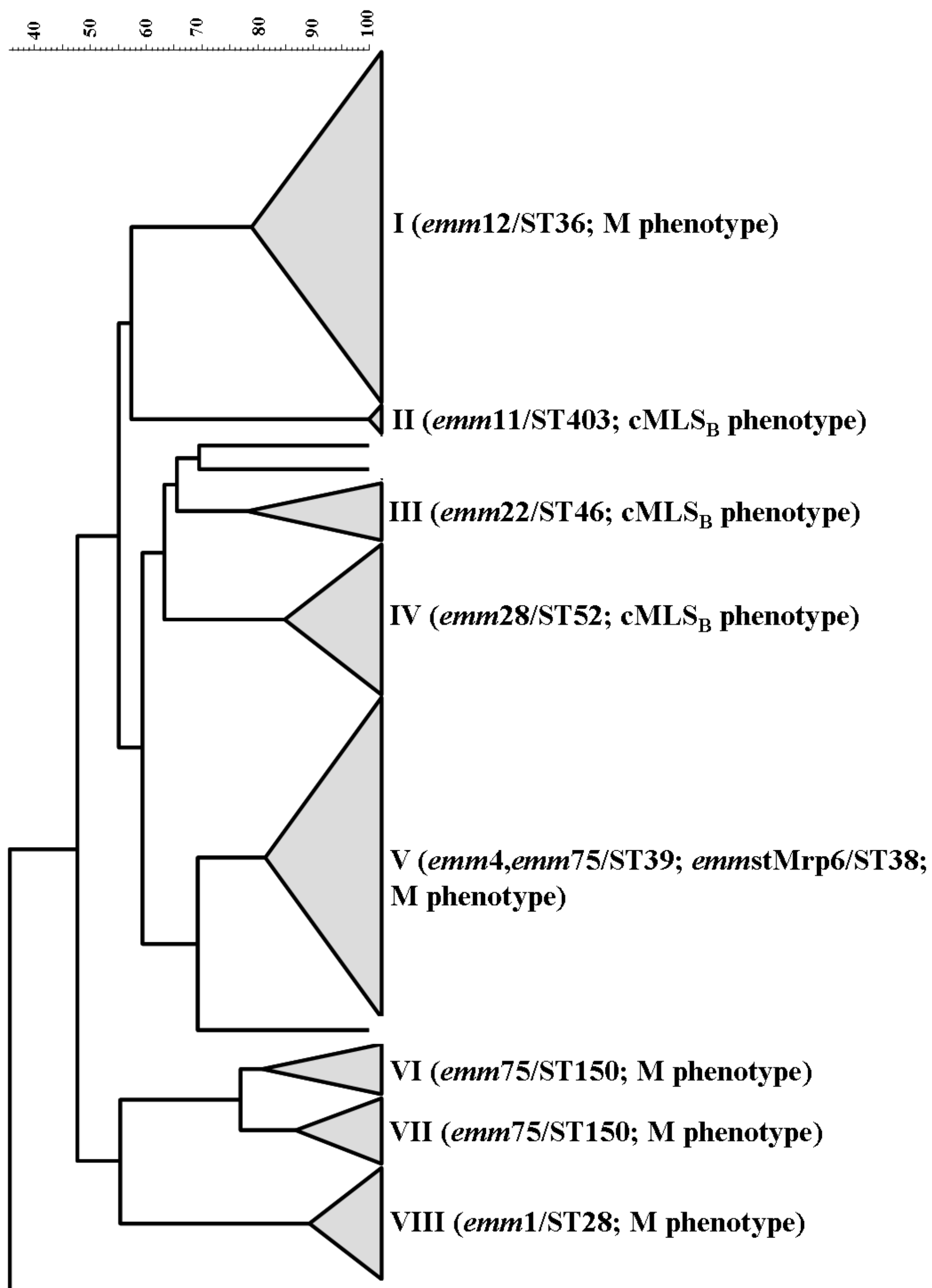

Figure 3.2. Dendrogram of the PFGE profiles of macrolide-resistant *S. pyogenes* from asymptomatic oropharyngeal colonization (2000-2006) in children. Dice coefficients are represented above the dendrogram. Major clonal lineages are indicated in bold romanic numerals.

Table 3.3. Characteristics of macrolide-resistant *S. pyogenes* isolates from oropharyngeal colonization (2000-2006) in children and distribution of clonal lineages by year of study.

Clonal lineage	No. of isolates (%)	Phenotype	T-types (no. of isolates)	*emm* types (no. of isolates)	ST (no. of isolates)	Year (no. of isolates) 2000	2001	2002	2003	2004	2005	2006
I	44 (29.5%)	M	12 (21), 5/11/12/27/44 (1); NT (22)	12 (44)	36 (5)	0	0	10	27	7	0	0
V	40 (26.8%)	M	4 (27), 8/25/Imp19 (1); NT (12)	4 (38), 75 (1), stMrp6 (1)	39(5), 38 (1)	0	1	0	20	19	0	0
IV	20 (13.4%)	$cMLS_B$	28 (20)	28 (20)	52 (5)	1	0	1	4	2	0	12
VIII	14 (9.4%)	M	1 (14)	1 (14)	28 (2)	2	11	1	0	0	0	0
VII	9 (6.0%)	M	8/25/Imp19 (5), 13 (1); NT (3)	75 (9)	150 (2)	0	0	0	6	3	0	0
III	7 (4.7%)	$cMLS_B$	12 (7)	22 (7)	46 (5)	1	0	0	6	0	0	0
VI	7 (4.7%)	M	8/25/Imp19 (6); NT (1)	75 (7)	150 (3)	0	0	1	6	0	0	0
II	4 (2.7%)	$cMLS_B$	11 (3); NT (1)	11 (4)	403 (2)	0	0	0	0	2	2	0
Other	4 (2.7%)	$cMLS_B$	12 (2), 6 (1); NT (1)	6 (1), 12 (1), 22 (1), 44 (1)	nd	1	1	0	0	2	0	0

Legend: NT – T nontypeable; nd – not done.

DISCUSSION

In this study we report the epidemiological and genotypic traits of macrolide-resistant *S. pyogenes* associated with asymptomatic oropharyngeal colonization during 2000 to 2006 in Lisbon area.

Our results showed that the frequency of resistance to macrolides among healthy pharyngeal carriers was variable along the seven-year study period (2000 to 2006), ranging from a maximum in 2003 (28%) to a minimum in 2005 (3%). Macrolide resistance among colonization isolates was also reported as variable in a few countries, ranging from very low (0-2%), such as in Japan (Iimura *et al.*, 2006) and Brazil (De Melo *et al.*, 2003), to very high in China (95%) (Chang *et al.*, 2010) and Italy (38%) (Creti *et al.*, 2005). In our study, the high macrolide resistance rates that we observed in 2003/2004 was paralleled by the rapid expansion of M phenotype isolates replacing the $cMLS_B$ phenotype isolates, which in turn replaced the M phenotype isolates in 2005, although with a low frequency. In 2005/2006, all the macrolide-resistant isolates associated with asymptomatic colonization were of the $cMLS_B$ phenotype. Temporal inversions of macrolide resistance phenotypes among colonization isolates were not reported previously and reinforce the importance of surveillance of carriers, as they may be indicators of the pool of isolates circulating in the community that may cause infections.

In fact, inversions of macrolide resistance phenotypes were detected among clinical isolates causing tonsillitis/pharyngitis in Portugal during the 2000-2006 period (Silva-Costa *et al.*, 2008). Interestingly, comparing both studies, we observed that the rise of the M phenotype was first detected in colonization isolates (in 2001; this study), and later in tonsillitis/pharyngitis isolates (in 2002) (Silva-Costa *et al.*, 2008). During these shifts, either in colonization (this study) or in tonsillitis/pharyngitis (Silva-Costa *et al.*, 2008) it was observed the prevalence of lineage *emm*1/ST28, which is not exclusively, although usually associated with severe *S. pyogenes* disease (Baldassarri *et al.*, 2007; Creti *et al.*, 2007; Friães *et al.*, 2007). Nevertheless, this lineage is frequently reported as being macrolide susceptible among patients with severe disease (Traverso *et al.*, 2010). On the contrary in 2005, when the $cMLS_B$ phenotype was dominant, the colonization isolates were *emm*11/ST403 (this study) whereas the tonsillitis/pharyngitis isolates were *emm*28/ST52 (Silva-Costa *et al.*, 2008). In 2006, the *emm*28/ST52 lineage was also dominant among the colonization $cMLS_B$ isolates. Studies including both colonization and infection isolates from comparable geographic locations and expanded time of isolation are therefore informative for global epidemiological analysis.

Our data showed that the macrolide-resistant isolates belonged to eight major lineages, defined by PFGE cluster/*emm*-type/ST. A strong association was found between PFGE and macrolide resistance phenotypes. Also, among the macrolide-resistant isolates, a strong

association was found between major PFGE clusters and prevalent T/*emm*/ST, despite the observed diversity of T/*emm*-types.

The fluctuation of macrolide resistance observed in the study period of 2000-2004 paralleled the fluctuation of M phenotype lineages, here defined as lineage I (*emm*12/ST36), VIII (*emm*1/ST28) – both homogeneous in terms of PFGE, *emm* type and ST –, and lineage V of one PFGE cluster and more than one *emm* type and ST (*emm*4, *emm*75/ST39; *emm*stMrp6/ST38), suggesting more than one strain in lineage V.

Each one of these lineages was detected during at least two consecutive years, indicating persistence among the total *S. pyogenes* population. *S. pyogenes* population of these sequence types, ST36, ST39 and ST28, has been described as associated with the upper respiratory tract and invasive infections in several European countries (Reinert *et al.*, 2004a; McGregor and Spratt, 2005; Aguero *et al.*, 2008; Silva-Costa *et al.*, 2008). It is unclear at this point whether these resistant strains are spreading worldwide or whether this represents multiple acquisitions of resistance genes by the prevalent *emm*/STs.

Among the $cMLS_B$ phenotype isolates, three lineages were found, all homogenous in terms of *emm*-types and ST: the most populated lineage IV (*emm*28/ST52), lineage II (*emm*11/ST403) and lineage III (*emm*22/ST46). The $cMLS_B$ phenotype isolates of lineage IV were resistant to bacitracin (Pires *et al.*, 2009). $cMLS_B$ isolates genetically related with the ones found in our study were already detected associated with oropharyngeal colonization in China (Chang *et al.*, 2010), as well as with pharyngitis in Europe (Mihaila-Amrouche *et al.*, 2004; Littauer *et al.*, 2006; Silva-Costa *et al.*, 2006; Ardanuy *et al.*, 2010), and also described as dominant among invasive isolates in Portugal (Friães *et al.*, 2005) and other countries (Malhotra-Kumar *et al.*, 2003; Grivea *et al.*, 2006). *emm*11/ST403 isolates (lineage II) were described for the first time among tonsillitis isolates in Spain (Ardanuy *et al.*, 2010) and in the present study was found for the first time among colonization isolates. *emm*22/ST46 isolates (lineage III) has already been described among oropharyngeal colonization elsewhere (Chang *et al.*, 2010) and in tonsillitis episodes from Portugal (Silva-Costa *et al.*, 2006) and Norway (Littauer *et al.*, 2006).

Regarding the virulence genotypes of the colonization isolates we have observed few statistically significant associations. Examples are *emm*1 (*spe*A$^+$, *ssa*$^-$), *emm*4 (*ssa*$^+$, *prtF1*$^+$) or *emm*12 (*speA*$^-$, *ssa*$^-$). The high prevalence (>20%) of *speC*, *prtF1* and *ssa*, in our study, was in most cases possibly due to lateral gene transfer events, i.e. the presence of a given gene was associated with more than one *emm*-type (or strain).

In particular, the *prtF1* gene was detected in 43% of the tested isolates, reinforcing the notion that strains carrying *prtF1* remain in the oropharynx of the host causing a carrier state (Molinari *et al.*, 1997). Moreover, it was suggested that M phenotype isolates that do not carry

prtF1 are poorly equipped to enter cells and may use the biofilm formation to escape antimicrobial treatments and surviving within the host (Baldassarri *et al.*, 2007).

As referred to above, high frequencies of macrolide resistance were observed in two distinct time periods, first associated with the presence of three M phenotype lineages, and thereafter due exclusively to a $cMLS_B$ multiresistant lineage. The reasons behind these major epidemiologic shifts remain to be clarified and whether or not they are related to selective pressure due to macrolide usage are also unclear. In fact, considering the data of antimicrobial consumption prior to sampling reported by our study population (see Table 3.1) we observed that macrolide consumption was not very high (0.7%). However, according to the European Surveillance of Antimicrobial Consumption project (ESAC), the consumption of macrolides, in particular, in Portugal has increased in 2000-2006 from 3.652 to 3.980 DID (http://www.esac.ua.ac.be/). Also, it was reported that the overall macrolide consumption fluctuated slightly over time, but a tendency to increase from 3.06 DID in 1997 to 3.81 DID in 2003 was noticed (Coenen *et al.*, 2006). Moreover, from 1997 to 2003, a gradual decrease in short-acting macrolides use from 1.26 DID to 0.29 DID in parallel with an increase in intermediate- and long-acting macrolides use from 1.35 DID to 2.14 DID and from 0.45 DID to 1.47 DID, respectively, was documented (Coenen *et al.*, 2006). This increase of macrolide consumption, specially of long-acting macrolides (like azithromycin), was probably the main cause for the rise in erythromycin resistance detected from 2000 to 2003, since compared with short- (like erythromycin) or intermediate-acting macrolides (like clarithromycin), long-acting macrolides may cause an enhanced resistance selection, because they selected quantitatively more resistant organisms in the early post-therapy phases, as suggested (Malhotra-Kumar *et al.*, 2007). However, it is not clear how the consumption of macrolides, in particular short-acting and long-acting macrolides could have influenced the fluctuation in resistance, hence in the phenotypes.

In conclusion, the genotypic properties of *S. pyogenes* from asymptomatic colonization described here contributes to a better understanding of the molecular epidemiology and evolution of specific strains and allowed to address the role of the pharyngeal carriers as potential vectors in the dissemination of clones into the community and also as reservoirs of macrolide resistance and virulence genes capable of being transferred to other commensal species sharing the same niche that cause associated diseases.

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Chapter IV

EMERGENCE OF CIPROFLOXACIN-NONSUSCEPTIBLE *STREPTOCOCCUS PYOGENES* FROM HEALTHY CHILDREN AND PEDIATRIC PATIENTS IN PORTUGAL

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With kind permission from American Society for Microbiology

ABSTRACT

We describe 66 ciprofloxacin-nonsusceptible *Streptococcus pyogenes* isolates recovered from colonized and infected children. The ParC-S79A substitution was frequent and associated with *emm*6/ST382 lineage. The ParC-D83G substitution was detected in two isolates (*emm*5/ST99 and *emm*28/ST52 lineages). One isolate (*emm*89/ST101) had no QRDRs codon substitutions or other resistance mechanisms. Five of 66 isolates were levofloxacin-resistant. Although fluoroquinolones are not used in children, they may be putative disseminators of fluoroquinolone-nonsusceptible strains in the community.

Keywords: Ciprofloxacin-nonsusceptibility, *Streptococcus pyogenes*, colonization, infection, lineages

TEXT

S. pyogenes clinical isolates with reduced susceptibility to fluoroquinolones (Albertí *et al.*, 2005; Alonso *et al.*, 2005; Malhotra-Kumar *et al.*, 2005; Orscheln *et al.*, 2005; Doloy *et al.*, 2008; Yan *et al.*, 2008; Smeesters *et al.*, 2009; Montes *et al.*, 2010) or with high-level resistance (Yan *et al.*, 2000; Richter *et al.*, 2003; Reinert *et al.*, 2004b; Rivera *et al.*, 2005; Malhotra-Kumar *et al.*, 2009; Montes *et al.*, 2010) have been described and the reduced susceptibility to fluoroquinolones is mediated by point mutations in the quinolone resistance-determining region (QRDR) of *parC* gene (Alonso *et al.*, 2005; Orscheln *et al.*, 2005; Montes *et al.*, 2010), whereas high-level resistance has been associated with mutations in the QRDRs of both *parC* and *gyrA* genes (Yan *et al.*, 2000; Reinert *et al.*, 2004b). To the best of our knowledge, there are no reports documenting the prevalence and characterization of fluoroquinolone-nonsusceptible *S. pyogenes* associated with asymptomatic colonization. Since 1999/2000, we have been collecting pediatric *S. pyogenes* isolates from different clinical origins and from carriers for the surveillance of antimicrobial susceptibility and for the epidemiological characterization of the isolates. This study aimed to describe the prevalence of ciprofloxacin-nonsusceptible *S. pyogenes* from colonized and infected Portuguese children from 1999 to 2006, and to characterize the associated clones and resistance mechanisms.

Strains and antibiotic susceptibility. A total of 1,354 nonduplicated *S. pyogenes* isolates were collected from children in Lisbon area, Portugal: 901 were associated with asymptomatic colonization during 2000-2006; 399 associated with tonsillitis/pharyngitis (2000-2006), 48 with skin/soft tissue infections (1999-2005), and six isolates were from invasive diseases (1999-2005). Identification was performed by standard methods (Pires *et al.*, 2005).

Susceptibility testing to ciprofloxacin was done to all isolates by disk diffusion (CLSI, 2008) using previously described breakpoints (Yan *et al.*, 2000). Figure 4.1 shows the strategy followed for the detection of ciprofloxacin-nonsusceptible isolates. MIC to ciprofloxacin was tested by agar dilution method (CLSI, 2008) (Fig. 4.1). MICs to other antimicrobial agents (Table 4.1) were determined by microdilution (CLSI, 2008), except for tigecycline (E-test strips were used).

Sixty-six out of the 1,354 isolates (4.9%) were considered as putative ciprofloxacin-nonsusceptible (MIC ≥2 µg/mL; range 2-8 µg/mL) (Fig. 4.1 and Table 4.1). Comparing by origin, ciprofloxacin nonsusceptibility was higher among clinical isolates (6.0%, n=27/453) than among carriage isolates (4.3%, n=39/901) (p=0.001). Similar rates were found among skin/soft tissue infection (6.3%, n=3/48) and tonsillitis/pharyngitis (6.0%, n=29/487) isolates (p=0.004), and no ciprofloxacin-nonsusceptible invasive disease isolates were detected (n=0/6) (p=0.13). To the best of our knowledge, this is the first study describing the incidence of ciprofloxacin-

nonsusceptibility among *S. pyogenes* collected from oropharyngeal colonization during a seven-year period (2000 to 2006). The rate of ciprofloxacin nonsusceptibility among our sample of clinical isolates was comparable to those previously reported among pediatric patients in Brazil, Belgium and USA (6% to 9%) (Yan *et al.*, 2008; Malhotra-Kumar *et al.*, 2009; Smeesters *et al.*, 2009) however it was slightly lower comparing with a study from Spain (13.6%) (Montes *et al.*, 2010) and much lower than the one found in Brussels (22.5%) (Smeesters *et al.*, 2009).

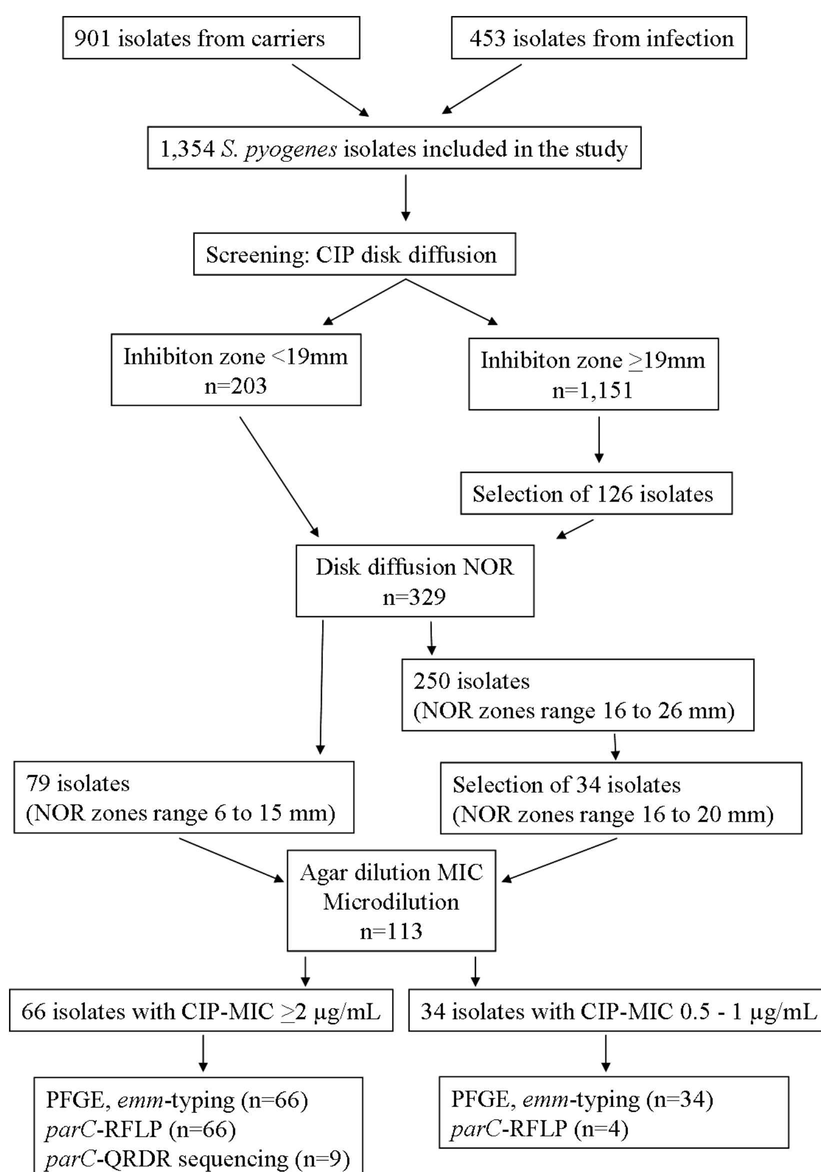

Figure 4.1. Diagram representing the methodologies used for the selection and characterization of ciprofloxacin-nonsusceptible *S. pyogenes*.

Legend: CIP, ciprofloxacin; NOR, norfloxacin.

Table 4.1. MICs to ciprofloxacin and 12 other antimicrobial agents and susceptibility rates among 66 ciprofloxacin-nonsusceptible *S. pyogenes* isolates collected from different origins in Portugal (1999-2006)[a].

Antimicrobial agent(s)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC range (µg/mL)	%S	%IR	%R
Ciprofloxacin	2	4	2 - 8	–[b]	–	–
Levofloxacin	2	2	2 - 4	92.4	7.6	0
Moxifloxacin	0.5	0.5	≤0.25 - 1	–	–	–
Erythromycin	≤0.25	≤0.25	≤0.25 - >32	98.5	0	1.5
Clindamycin	≤0.25	≤0.25	≤0.25 - >5	98.5	0	1.5
Penicillin	≤0.03	≤0.03	≤0.03	100	0	0
Ampicillin	≤0.25	≤0.25	≤0.25	100	0	0
Cefotaxime	≤0.06	≤0.06	≤0.06	100	0	0
Vancomycin	0.5	0.5	≤0.25 - 0.5	100	0	0
Teicoplanin	≤0.25	≤0.25	≤0.25	100	0	0
Quinupristin/dalfopristin	≤1	≤1	≤1	100	0	0
Tetracycline	≤2	≤2	≤2 - >4	98.5	0	1.5
Tigecycline	0.047	0.064	0.025 - 0.064	–	–	–

Legend: [a] Susceptibility breakpoints according to CLSI interpretative criteria (CLSI, 2008). S, susceptible; IR, intermediate resistant; R, resistant. [b] –, No interpretative criteria.

Overall, ciprofloxacin-nonsusceptible isolates (n=66) presented high rates of susceptibility (>90%) to other antimicrobial agents (Table 4.1). Nonsusceptibility to levofloxacin was 7.6% (n=5/66). One isolate was resistant to both erythromycin and clindamycin and presented the $cMLS_B$-macrolide resistance phenotype and *erm*(B) genotype, detected by PCR (Pires *et al.*, 2005). Another isolate was tetracycline-resistant carrying *tet*(M) and *tet*(T) and not *tet*(K), *tet*(L), *tet*(O), *tet*(Q), *tet*(S), or *tet*(W) genes, according to PCR screening assays (Pires *et al.*, 2005; Rato *et al.*, 2010).

Molecular typing. The relationship among all the ciprofloxacin-nonsusceptible isolates was assessed by PFGE (Pires *et al.*, 2009). Detection of *emm*-types was carried out as described (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm) and representative isolates of different PFGE pattern/*emm*-type associations (n=9) were analyzed by MLST (http://spyogenes.mlst.net).

The 66 ciprofloxacin-nonsusceptible isolates were included in seven different PFGE patterns, arbitrarily named with capital letter codes (Table 4.2).

Table 4.2. Genotypes, phenotypes and origins of the 66 ciprofloxacin-nonsusceptible *S. pyogenes* isolates collected in Portugal (1999-2006).

PFGE pattern (No. isolates)	*emm*-type (No. isolates)	Sequence type [a]	MIC range (µg/mL) [b]			CIP and LVX co-resistance (No. isolates)	Aminoacid change in ParC [c]		Origin [e] (No. isolates)
			CIP	LVX	MXF		S79	D83	
AD (58)	*emm*6 (52)	ST382	2-8	2-4	≤0.25-0.5	2	A	- [d]	OC (30), T/P (20), S/STI (2)
	*emm*1 (5)	ST382	2-4	2-4	0.5	1		-	OC (2), T/P (3)
	*emm*89 (1)	ST382	4	4	1	1	A	-	OC (1)
DX (2)	*emm*6 (2)	ST382	2-4	2	0.5	0	A	-	OC (2)
DY (2)	*emm*6 (2)	ST382	4	2-4	0.5-1	1	A	-	OC (2)
AM (1)	*emm*6 (1)	ST382	2	2	0.5	0	A	-	OC (1)
BT (1)	*emm*28 (1)	ST52	2	2	≤0.25	0	-	G	S/STI (1)
CJ (1)	*emm*89 (1)	ST101	2	2	0.5	0	-	-	T/P (1)
K (1)	*emm*5 (1)	ST99	2	2	0.5	0	-	G	OC (1)

Legend: [a] Only one isolate of each PFGE/*emm* association was analyzed by MLST. [b] CIP – ciprofloxacin, LVX – levofloxacin, MXF – moxifloxacin. [c] The *parC* gene was sequenced in selected isolates (n=9). The remaining isolates had mutation detected by PCR-RFLP. The *emm*89/ST101 isolate presented the triple substitution D91N/S107L/S140P and did not present any *gyrA, gyrB* or *parE* mutation (see the text). [d] no change. [e] OC – oropharyngeal colonization; T/P – tonsillitis/pharyngitis; S/STI – skin/soft tissue infection.

PFGE pattern AD was observed in 87.9% of the isolates (n=58/66), which were mainly *emm*6 (n=52). Three other PFGE patterns (DX, DY and AM) also included *emm*6/ST382 isolates (n=5). PFGE-AD, PFGE-DX, PFGE-DY and PFGE-AM were grouped into the same lineage – ST382 – which included 95.5% of the ciprofloxacin-nonsusceptible isolates (n=63/66). The remaining three PFGE patterns included single isolates: PFGE-BT (*emm*28/ST52 lineage), PFGE-CJ (*emm*89/ST101 lineage) and PFGE-K (*emm*5/ST99 lineage). The origins of the isolates are shown in Table 4.2.

GenBank accession number

The sequence of the new *emm*-subtype 6.63 was submitted to GenBank database under the accession number FJ711064.

Quinolone resistance characterization. Detection of point mutations in the *parC* quinolone resistance-determining region (QRDR) was performed by PCR (Rivera *et al.*, 2005; Biedenbach *et al.*, 2006) and by restriction fragment length polymorphism (RFLP) (Alonso *et al.*, 2004) (Figure 4.1).

All but one ciprofloxacin-nonsusceptible isolates had *parC*-QRDR mutations generating the amino acid substitutions S79A (n=63) and D83G (n=2).

The ParC-S79A substitution was found among clinical and colonization ciprofloxacin-nonsusceptible isolates of *emm*6 (n=57), *emm*1 (n=5) and *emm*89 (n=1) of the major lineage ST382 (see Table 4.2).

Substitutions in codon S79 of ParC QRDR have been found as prevalent in *emm*6 fluoroquinolone-resistant *S. pyogenes* isolates (Albertí *et al.*, 2005; Alonso *et al.*, 2005; Malhotra-Kumar *et al.*, 2005; Orscheln *et al.*, 2005; Yan *et al.*, 2008; Montes *et al.*, 2010) and considered to be intrinsic (Orscheln *et al.*, 2005) and not related with selective pressure by antibiotic usage.

While *emm*89 was previously found among sporadic ciprofloxacin-nonsusceptible strains (Reinert *et al.*, 2004b; Doloy *et al.*, 2008), *emm*1 was associated for the first time in this study with fluoroquinolone nonsusceptibility.

The ParC-D83G substitution was also found for the first time in this study, particularly among isolates of lineages *emm*28/ST52 (n=1) and *emm*5/ST99 (n=1) and was previously described in *S. pneumoniae* (Pletz *et al.*, 2006).

We also detected these two latter ciprofloxacin-nonsusceptible lineages, together with lineage *emm*22/ST46 with D83G/Y substitutions, among infection products from adults (data not shown). This D83Y codon replacement was previously associated with nonsusceptibility to fluoroquinolones among other streptococci (Biedenbach *et al.*, 2006; de la Campa *et al.*, 2009).

No ParC substitutions at S79 or D83 codons were detected in the *emm*89/ST101 isolate but instead replacements were observed at D91, S107 and S140 codons (Table 4.2). This isolate was also investigated for the presence of mutations in the QRDRs of *gyrA*, *gyrB* and *parE*, as described (Biedenbach *et al.*, 2006; Rivera *et al.*, 2005), and no mutations were found. Moreover, plasmid encoded specific genes *qnrA*, *qnrB*, *qnrC* nor *aac(6')-Ib-cr* that confer resistance to fluoroquinolones in *Enterobacteriaceae* were not detected after PCR screening assays (Brisse and Verhoef, 2001; Lévesque *et al.*, 2005). Also, MIC of ciprofloxacin, levofloxacin and moxifloxacin did not decreased after the addition of 10 µg/mL of reserpine, which is an efflux pump inhibitor (Ferrándiz *et al.*, 1999). Substitutions at D91 and S140 positions are most likely not involved in fluoroquinolone resistance because they can be found in both susceptible and resistant isolates (Duesberg *et al.*, 2008). Similarly, the substitution at S107 position, which was not previously reported in *S. pyogenes*, may also not be involved in fluoroquinolone resistance because it was detected in one ciprofloxacin-susceptible isolate of our study (data not shown).

Fluoroquinolones have been widely used in Portugal for many years (Ferech *et al.*, 2006) and continuous use has been implicated in selection for resistance (Malhotra-Kumar *et al.*, 2005). Particularly, older fluoroquinolones, like ciprofloxacin, were previously suggested to promote dissemination of fluoroquinolone nonsusceptibility among different *S. pyogenes* clones (Albertí *et al.*, 2005; Malhotra-Kumar *et al.*, 2005). On the other hand, horizontal genetic exchange by interspecies recombination has also been reported as leading to quinolone resistance in *S. pyogenes* (Duesberg *et al.*, 2008).

The finding of a few ciprofloxacin nonsusceptible strains, other than *emm*6, may suggest the occurrence of horizontal gene transfer involving the *parC*-QRDR region. The recognition of ciprofloxacin nonsusceptibility among isolates colonizing healthy children attending day-care centers and schools highlights their possible role as disseminators of ciprofloxacin-nonsusceptible strains, as it has been recognized for other bacteria (Sá-Leão *et al.*, 2008).

In conclusion, our study demonstrated that in Portugal fluoroquinolone resistance in pediatric *S. pyogenes* is mediated by both clonal dissemination and unrelated events of development of resistance. Whether or not high and sustained fluoroquinolone consumption in Portugal has been implicated in selection for resistance in *S. pyogenes*, the recognition of colonization and clinical isolates nonsusceptible to ciprofloxacin, particularly in adult populations should be a cause of concern and may compromise the therapeutic importance of these antimicrobials. A continuous surveillance of fluoroquinolone resistance is important to monitor the evolution of nonsusceptible *S. pyogenes* isolates.

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All the experimental work was performed by R. Pires, with the exception of some sequencing data that were done by Vera Oliveira and preliminary characterization of the isolates carried out by graduate and undergraduate students of the laboratory.

Chapter V

RESISTANCE TO BACITRACIN IN *STREPTOCOCCUS PYOGENES* FROM OROPHARYNGEAL COLONIZATION AND NONINVASIVE INFECTIONS IN PORTUGAL WAS CAUSED BY TWO CLONES OF DISTINCT VIRULENCE GENOTYPES

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ABSTRACT

During 2000-2007 in Lisbon, we identified 45 bacitracin-resistant *Streptococcus pyogenes* isolates among 1,629 isolates: 24 from oropharyngeal healthy carriers (out of 1,026), 21 from patients with noninvasive infections (out of 559) and zero from invasive infections (out of 44). Forty-four of those isolates, mainly of colonization, are low-level bacitracin-resistant and members of the $cMLS_B$-macrolide-resistant and tetracycline-susceptible *emm*28/ST52 clone, previously detected in Europe but only among clinical samples. One high-level bacitracin resistant isolate, associated with a tonsillitis/pharyngitis episode, is $cMLS_B$-macrolide-resistant and tetracycline-resistant and a member of the *emm*74/ST120 lineage, which was not previously known to include bacitracin-resistant isolates. The *bcrABDR* operon encoding an ATP-binding cassette (ABC) transporter in *Enterococcus faecalis* was not detected among these bacitracin-resistant *S. pyogenes* strains. Virulence profiling indicated that genes coding for exotoxins and superantigens seem to be clone specific. This study provides an increased knowledge about specific bacitracin-resistant *S. pyogenes* strains which may be useful in future investigations aiming to understand the mechanism(s) leading to bacitracin resistance and the cause(s) for differences in colonization and/or dissemination potential.

Keywords: *Streptococcus pyogenes*, colonization, infection, bacitracin, resistance, virulence.

INTRODUCTION

Streptococcus pyogenes (Group A *Streptococcus* – GAS) are among the most common pathogenic bacteria that infect children and adolescents and are associated with a wide variety of infections and disease states, ranging from uncomplicated but highly prevalent pharyngitis to extremely severe infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome (Cunningham, 2000).

One of the presumptive tests for *S. pyogenes* identification is the susceptibility to bacitracin, which provides the differentiation from other beta-hemolytic streptococci of human origin (Facklam, 2002). However, clinical isolates resistant to bacitracin have been documented in the literature (Pérez-Trallero *et al.*, 2007).

Bacitracin is involved in both binding and sequestering of the undecaprenol pyrophosphate (UPP), the precursor of undecaprenol monophosphate (UP), a lipid carrier involved in cell wall synthesis. Usually, ATP-binding cassette (ABC) transporters that mediate active efflux of bacitracin confer high-level resistance to. Among the bacitracin-producing strains of *Bacillus licheniformis*, this ABC transporter is encoded by *bcrABC* genes (Podelsek *et al.*, 1995). A homologue of this transporter, encoded by *mbrABCD* genes, was also found among the intrinsic bacitracin-resistant species *Streptococcus mutans* (Tsuda *et al.*, 2002). A putative homodimeric ABC transporter encoded by the *bcrABD* genes was found to confer resistance to bacitracin in *Enterococcus faecalis* (Manson *et al.*, 2004). Overexpression of the *bacA* gene, which leads to the overproduction of undecaprenol kinase, seem to increase the cellular amount of the lipid carrier, and consequently to increase the resistance of the organism to bacitracin (Cain *et al.*, 1993). Also, allelic replacement mutagenesis of *bacA* gene of strains of other Gram positive bacteria caused an increased susceptibility to bacitracin, further suggesting that the *bacA* gene product is essential for the resistance to this antibiotic (Chalker *et al.*, 2000).

In this study we aimed to select and further characterize bacitracin-resistant *S. pyogenes* collected from oropharyngeal carriers and patients with diagnosed infections, during 2000 to 2007 in Portugal, to know the prevalence of bacitracin resistance among isolates from colonization and disease, to evaluate if the bacitracin resistance mechanism in *S. pyogenes* was similar to the one described in *E. faecalis*, to assess the putative variability of genetic backgrounds and to provide new data concerning virulence genotypes of the isolates, in particular genes coding for exotoxins, superantigens and invasins.

MATERIALS AND METHODS

Origin of the *S. pyogenes* isolates

A total of 1,629 nonduplicated *S. pyogenes* isolates associated with colonization and symptomatic infections were collected from children and adults in 20 educational and eight health-care institutions (15 day-care centers and five schools; five ambulatories and three hospitals) located in Lisbon area, Portugal, during a surveillance study carried out from 2000 to 2007, for comparison of *S. pyogenes* from colonization and disease (Pires *et al.*, 2005; Santos-Sanches *et al.*, unpublished). Out of the 1,629 isolates, 1,026 were recovered from 10,578 throat swabs of asymptomatic populations (children and adults) during 2000-2007 (average colonization rate of 9.7%) and 603 isolates were from patients diagnosed with clinical infections: 487 with tonsillitis/pharyngitis in 2000-2006, 72 with skin/soft tissue infections in 1999-2005, and 44 with invasive diseases (all isolates from usually sterile sites) in 1999-2005. The isolates were identified by beta-hemolysis and colony morphology on sheep-blood agar, agglutination with group A-specific antiserum (Slidex Strepto, bioMérieux™, Marcy l'Etoile, France) and detection of PYR (L-pyrrolidonil-β-naphthylamide - DrySlide™, Becton Dickinson, Le Pont de Claix, France).

Selection of bacitracin-resistant isolates and searching for resistance genotypes

Susceptibility to bacitracin was carried out by disk diffusion, using a 0.04 U bacitracin-containing disk (BBL™, Becton Dickinson, Le Pont de Claix, France). The isolates with no inhibition zone around the bacitracin disk were interpreted as bacitracin-resistant (Pérez-Trallero *et al.*, 2004) and were selected for this study. Minimum inhibitory concentrations (MIC) were evaluated by agar dilution on Mueller-Hinton agar supplemented with 5% sheep blood, with the following concentrations of bacitracin: 0.5, 1, 2, 4, 8, 16, 32, 64 and 256 mg L^{-1}. The control strains used were the *Escherichia coli* DH5-α carrying the p2H7 plasmid containing *bcrABDR* operon of *E. faecalis* (provided by M. F. Lopes), which confers resistance to bacitracin, and two *S. pyogenes* isolates identified as bacitracin-susceptible by disk diffusion. Isolates were tested for the presence of *bcrABDR* operon by PCR, using *E. coli* DH5-α carrying p2H7 plasmid as positive control. Briefly, template DNAs were obtained as described (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). The primers used were as described (Matos *et al.*, 2009). Expected sizes for *bcrABDR* PCR products were 584, 489, 482 and 461 bp, respectively. The amplification PCR reactions (50 µl) included 1 µl of template DNA, 1 U of *Taq* DNA Polymerase (Invitrogen, Carslbad, USA), 1× Reaction Buffer, 3 mM $MgCl_2$, 200 µM deoxynucleotides and 0.2 µM of each primer. Amplifications were carried out in a temperature-gradient thermocycler (T Gradient, Biometra®, Goettingen, Germany), with the following conditions: initial denaturation at 95ºC (5 min); 30 cycles of denaturation at 95ºC (30 s),

annealing at 51ºC (45 s) and polymerization at 72ºC (30 s); final polymerization at 72ºC (5 min) (Matos *et al.*, 2009).

Comparison of bacitracin resistance frequency among *S. pyogenes* isolates from colonization and symptomatic infections

Chi-square test was used to test significance of the observed differences in the standardized frequency of resistance to bacitracin among isolates from colonization and of the different clinical origins.

Macrolide, lincosamide and tetracycline phenotypes and genotypes

All bacitracin-resistant isolates were tested for susceptibility to macrolides (erythromycin, azithromycin, clarithromycin), lincosamides (clindamycin) and tetracycline by disk diffusion (Oxoid Ltd., Basingstoke, UK), according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2008). The MICs to erythromycin, clindamycin and tetracycline were evaluated by E-test (AB Biodisk®, Solna, Sweden), according to the manufacturer's instructions. Resistance to 14- and 15- membered macrolides (M phenotype) or to macrolides, lincosamides and streptogramin B, either inducible ($iMLS_B$) or constitutive ($cMLS_B$), were determined by the double-disk test with erythromycin and clindamycin (Seppälä *et al.*, 1993). The macrolide resistance genes *mef*(A), *erm*(A) and *erm*(B), and the tetracycline resistance gene *tet*(M) were searched for by PCR as described (Pires *et al.*, 2005).

Pulsed-field gel electrophoresis (PFGE)

The relationship among all the bacitracin-resistant isolates was assessed by PFGE. Chromosomal DNA preparation and DNA digestion with *Sma*I (New England Biolabs®, Beverly, USA) was performed essentially as described previously (Chung *et al.*, 2000), except the composition of the lysis buffer which consisted of 50 µg of RNase I, 1 mg of lysozyme, and 5 U of mutanolysin in 1 mL of lysis buffer (6 mM Tris, pH 8, 1 M NaCl, 0.1 M EDTA, pH 8, 0.2% deoxycholate, 0.5% sarkosyl, 0.5% Brij 58). Running was performed in a contour-clamped homogeneous electric field system (CHEF-DRIII; Bio-Rad, Hemel Hempstead, UK). The running parameters were as described (Chung *et al.*, 2000). DNA-band analysis was performed by visual inspection. Since the number of DNA fragments was frequently less than 10, a single band difference was used as a criterion to define a different pattern, as suggested (Tenover *et al.*, 1995). The relationship among all patterns was assessed in a dendrogram generated by the BioNumerics® software version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium). Resemblance was computed with Dice similarity coefficient and agglomerative clustering was performed with the unweighted pair group method with arithmetic mean (UPGMA), with optimization and tolerance values of 1% and 1.5%, respectively.

T-serotyping, *emm*-typing and multilocus sequence typing (MLST)

A subset of isolates representatives of different PFGE patterns was selected for assignment of T capsular serotypes (T-types) (n=29 isolates), *emm* gene sequence types (*emm*-types) (n=22) and sequence types (ST) (n=8) by MLST.

T-types were determined by slide agglutination using 5-polyvalent and 21-monovalent anti-T-agglutination sera (Sevapharma, Prague, The Czech Republic) and *emm*-types were identified by *emm*-typing, as described by the *Streptococcus* laboratory from the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Internal fragments of seven housekeeping alleles (*gki*, *gtr*, *mur*I, *mut*S, *rec*P, *xpt* and *yqi*L) were used for identification of ST, as described at *S. pyogenes* MLST database (http://spyogenes.mlst.net/).

Virulence genotyping

Virulence profiles were determined after PCR detection of the *speA*, *speC*, *speF*, *speG*, *speH* genes, encoding streptococcal pyrogenic exotoxins, the *ssa* gene, encoding a superantigen, and the *prtF1* gene, encoding a fibronectin-binding protein (Jasir *et al.*, 2001; Schmitz *et al.*, 2003). The chromosomal *speF* and *speG* genes were used as positive controls. Negative results were consistent in three independent PCR assays using the appropriate controls.

RESULTS AND DISCUSSION

Bacitracin resistance in *S. pyogenes*

Forty-five out of 1,629 isolates (2.8%) were resistant to bacitracin. Forty-four of those isolates had a MIC of 16 mg L^{-1} and were considered as low-level bacitracin-resistant; the remaining isolate was highly resistant to bacitracin with a MIC of 256 mg L^{-}. The MIC of the bacitracin-resistant control strain was higher than 256 mg L^{-1} and of the two bacitracin-susceptible control strains was 1 mg L^{-1}. We found that the isolates, either high-level or low-level resistant to bacitracin, did not carry the *bcrABDR* operon, encoding an ABC transporter that was considered to confer high-level bacitracin resistance in *E. faecalis* (Manson *et al.*, 2004).

Frequency of bacitracin resistance in different origins

The proportion of bacitracin-resistant isolates in relation to origin was not significantly different (p>0.05): 24 out of 1,026 isolates from colonization (2.3%), 17 out of 487 from tonsillitis/pharyngitis (3.5%) and 4 out of 72 from skin/soft tissue infection (5.6%). No bacitracin-resistant isolates were identified among the 44 invasive isolates. Previous studies that documented antimicrobial resistance patterns of clinical isolates of *S. pyogenes* have also included data of bacitracin resistance (York *et al.*, 1999; Malhotra-Kumar *et al.*, 2003; Mihaila-Amrouche *et al.*, 2004; Pérez-Trallero *et al.*, 2004, 2007; Silva-Costa *et al.*, 2006). However, no data was previously published regarding bacitracin resistance among *S. pyogenes* colonization isolates. Also, to our best knowledge, only one study referred to bacitracin resistance in invasive isolates (7%, n=11/157 isolates); this study included isolates mainly collected in health institutions from San Francisco Bay Area, USA (York *et al.*, 1999). Among noninvasive isolates (mostly tonsillitis/pharyngitis), the highest frequency of bacitracin resistance was found in France (12%) (Mihaila-Amrouche *et al.*, 2004). In contrast, and comparing with our study, slightly lower rates have been reported in Europe: Belgium (1.3%, n=16/1,229) (Malhotra-Kumar *et al.*, 2003) and Spain (0.7%, n=115/17,232) (Pérez-Trallero *et al.*, 2007).

Resistance to macrolides, lincosamides and tetracycline

All bacitracin-resistant isolates were resistant to macrolides of the $cMLS_B$ phenotype. The erythromycin and clindamycin MICs were higher than 256 mg L^{-1}. All isolates carry the *erm*(B) gene and lack *erm*(A); one isolate carries in addition *mef*(A). The isolate with high-level bacitracin resistance was also resistant to tetracycline (MIC=64 mg L^{-1}) and was the unique *tet*(M)-positive. Resistance to bacitracin associated with macrolide resistance of the $cMLS_B$ phenotype was first reported in the USA, among isolates also resistant to tetracycline, collected during 1994-1995, but the genotypes of those isolates were not described (York *et al.*, 1999). In

Europe, bacitracin resistance has been described as associated with the $cMLS_B$ phenotype-*erm*(B) genotype (Malhotra-Kumar *et al.*, 2003; Mihaila-Amrouche *et al.*, 2004; Pérez-Trallero *et al.*, 2004, 2007; Silva-Costa *et al.*, 2006) but the isolates were susceptible to tetracycline (Mihaila-Amrouche *et al.*, 2004; Pérez-Trallero *et al.*, 2004, 2007). The reason why bacitracin resistance is only found among specific macrolide-resistant *S. pyogenes* of $cMLS_B$ phenotype rather than among macrolide-resistant isolates of M phenotype remains unclear.

Genomic backgrounds and genetic lineages

The 45 bacitracin-resistant isolates of the three origins were of six different PFGE patterns, arbitrarily named with capital letter codes (F, AK, AC, Q, BT and BU) (Figs. 5.1 and 5.2).

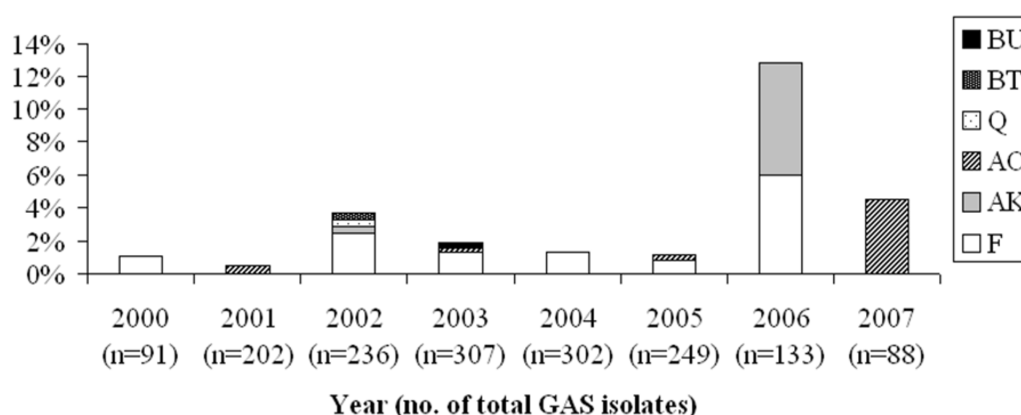
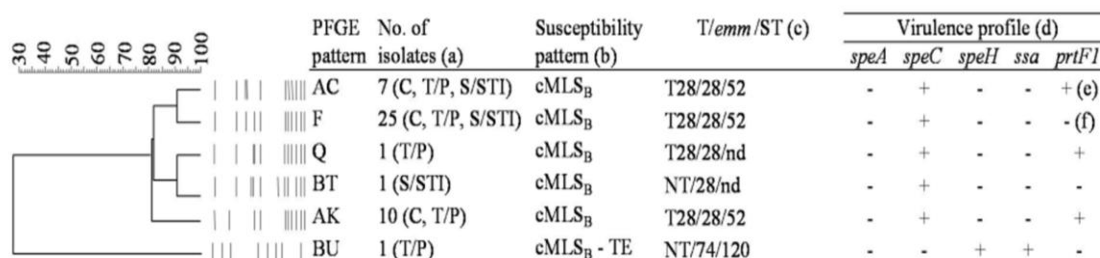


Figure 5.1. Annual distribution of PFGE patterns of the 45 bacitracin-resistant *S. pyogenes* isolates.

Capital letters are arbitrary denominations of PFGE-*Sma*I-macrorestriction patterns.

PFGE pattern F was persistent in the study period (n=25 isolates, 55.6%). PFGE-AK (n=10 isolates; 22.2%) and PFGE-AC (n=7; 15.6%) were less common, however PFGE-AK prevailed during 2006, and PFGE-AC accounted for all bacitracin-resistant isolates collected in 2007. PFGE-Q, PFGE-BU and PFGE-BT were unique patterns. Five of the six PFGE patterns (F, AK, AC, Q and BT) were considered related (>80% similarity) and of a single clonal group, whereas PFGE-BU was considered as distinct (30% similarity) and of another clonal group (Fig. 5.2). In most cases, the related isolates (>80% similarity) were T28 [except one T nontypeable (NT) isolate of PFGE-BT], *emm*28 and ST52. The 44 T28 or NT/*emm*28/ST52 isolates were also tetracycline-susceptible and low-level bacitracin-resistant. The isolate with the distinct pattern, PFGE-BU, of lineage NT/*emm*74/ST120 was tetracycline-resistant and high-level bacitracin-resistant.



PFGE pattern	No. of isolates (a)	Susceptibility pattern (b)	T/*emm*/ST (c)	*speA*	*speC*	*speH*	*ssa*	*prtF1*
				Virulence profile (d)				
AC	7 (C, T/P, S/STI)	$cMLS_B$	T28/28/52	-	+	-	-	+ (e)
F	25 (C, T/P, S/STI)	$cMLS_B$	T28/28/52	-	+	-	-	- (f)
Q	1 (T/P)	$cMLS_B$	T28/28/nd	-	+	-	-	+
BT	1 (S/STI)	$cMLS_B$	NT/28/nd	-	+	-	-	-
AK	10 (C, T/P)	$cMLS_B$	T28/28/52	-	+	-	-	+
BU	1 (T/P)	$cMLS_B$ - TE	NT/74/120	-	-	+	+	-

Figure 5.2. Properties of the bacitracin-resistant *S. pyogenes* from asymptomatic oropharyngeal colonization and infection sites in Portugal (2000-2007).

Dendrogram of the pulsed-field gel electrophoresis (PFGE) profiles using Dice coefficient and unweighted pair-group method with arithmetic mean clustering (BioNumerics software). Dice coefficients are represented above the dendrogram. Capital letters, PFGE-*Sma*I-macrorestriction patterns. [a] Origin of the isolates is indicated in parentheses: C, colonization; T/P, tonsillitis/pharyngitis; S/STI, skin/soft-tissue infections. [b] $cMLS_B$: constitutive resistance to macrolide-lincosamide-streptogramin B; TE: resistance to tetracycline. [c] T capsular type (T-type); *emm* gene sequence type (*emm*-type); sequence type (ST) by MLST; nd: not done; NT, nontypeable by anti-T-agglutination sera. [d] – negative by PCR; + positive by PCR. [e] One isolate lacks the *prtF1* gene. [f] One isolate carries the *prtF1* gene.

Infection isolates of T28/*emm*28 types, of the $cMLS_B$ phenotype and resistant to bacitracin were also found in France, associated with pharyngitis (2000-2001) and invasive infections (2000-2003) (Mihaila-Amrouche *et al.*, 2004), and in Belgium, associated only with pharyngitis (Malhotra-Kumar *et al.*, 2003). No MLST data were reported in those studies. Noninvasive isolates with the $cMLS_B$ phenotype, resistant to bacitracin and sharing the same properties (T28/*emm*28/ST52) with the isolates described here, were reported in Spain (1999-2005) (Pérez-Trallero *et al.*, 2004; 2007) and in Portugal (1999-2003) (Silva-Costa *et al.*, 2006). This T28/*emm*28/ST52 lineage is known to include bacitracin-resistant and bacitracin-susceptible isolates (Pérez-Trallero *et al.*, 2007), and isolates from diverse infection sites (throat, skin, blood) of different geographic locations (Europe, USA, Canada, South Korea) (http://spyogenes.mlst.net, last search at April 23, 2009).

The persistence of the T28/*emm*28/ST52 bacitracin-resistant lineage among oropharyngeal carriers during six years (2000, 2002-2004, 2006, 2007), as described in the present work, indicate that carriers are reservoirs of this particular clone of high epidemic potential and capable of a wide geographic dissemination. In contrast, the second bacitracin-resistant lineage, NT/*emm*74/ST120, was not detected among carriers in our study and according to the *S. pyogenes* MLST database, this lineage seem to have a limited geographic distribution (Australia, Nepal, India) (http://spyogenes.mlst.net, last search at April 23, 2009).

GenBank accession number

The sequence of the new *emm*-subtype 28.9 was submitted to GenBank database under the accession number FJ711061.

Virulence genotypes

Virulence profiles for exotoxins and superantigens seem to correlate with *emm*-type/ST but, in contrast, variable results were observed for the *prtF1* invasin gene (Fig. 5.2). The $cMLS_B$ isolates of *emm*28/ST52 carried *speC* gene. The presence of this particular gene has been reported as frequent among *emm*28 isolates from both noninvasive (86%) and invasive disease (75%) (Ekelund *et al.*, 2005), however no information regarding antimicrobial susceptibility patterns was referred to in that study. The $cMLS_B$ isolate of *emm*74/ST120 lineage did not carried *speC* but instead carried the *speH* and *ssa* genes. All the isolates of the two $cMLS_B$ bacitracin-resistant lineages lack *speA*, a gene frequently found among macrolide-resistant isolates of M phenotype (Creti *et al.*, 2007). Both *speC* and *speA* genes encoding the SpeC and SpeA pyrogenic exotoxins are located on genetic mobile elements and strains carrying these virulence determinants are usually associated with rheumatic fever episodes (Sriskandan, 2007); interestingly, the *speC*-positive isolates from our study were from diverse origins: colonization, throat infections and skin/soft-tissue infections. Few reports have documented the virulence traits of macrolide-resistant/*emm*28 isolates (Creti *et al.*, 2007) or of isolates of the ST52 lineage (Friães *et al.*, 2007), although in these reports no information is available regarding bacitracin resistance.

CONCLUSION

Altogether these data reinforce the limited accuracy of the bacitracin susceptibility test for *S. pyogenes* identification at the clinical setting. However, identification of these isolates is not useless as it allows monitoring specific epidemic clones and putative novel emergent clones. We report for the first time the long term persistence among carriers of the bacitracin-resistant *emm*28/ST52 lineage, which is prevalent in Europe. We also report for the first time a high-level bacitracin resistant isolate of the *emm*74/ST120 lineage, which was not previously known to include bacitracin-resistant isolates. We also show that the ABC transporter encoded by the *bcrABDR* operon may not be associated with bacitracin resistance among *S. pyogenes.* Further investigation will contribute to elucidate the cause(s) for the emergence, persistence and differences in colonization potential of bacitracin-resistant isolates and to ascertain the genetic basis for bacitracin resistance, particularly in the *emm*28/ST52 and *emm*74/ST120 strains described here.

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All the experimental work was performed by R. Pires, with the exception of the identification and characterization of part of the isolates which was carried out with the collaboration of graduate and undergraduate students of the laboratory.

Chapter VI

NON-RANDOM DISTRIBUTION OF VIRULENCE GENES IN GROUP A *STREPTOCOCCUS* (GAS) FROM COLONIZATION AND INFECTION

Part of this work was included in the Master Thesis entitled "Factores de virulência em *Streptococcus pyogenes*" (L. M. Lino, 2010). Part of these results were presented at XVIII Lancefield, September 4-8, 2011, Palermo, Italy.

ABSTRACT

Streptococcus pyogenes or Group A *Streptococcus* (GAS) is a Gram positive bacterium that can cause human diseases ranging from mild throat and skin infections to life-threatening diseases, such as toxic shock syndrome and necrotizing fasciitis (Cunningham, 2000). Asymptomatic carriers harbour the organism mainly in the throat or nose and display no symptoms of acute infection (Pichichero *et al.*, 1999). GAS produces a variety of extracellular products that are involved in the pathogenesis of various human infections (Cunningham, 2000). Previous studies have been carried out by our group using GAS from oropharyngeal colonization and from diverse infections, particularly for comparison of clones and antimicrobial resistance (Pires *et al.*, 2005; Pires *et al.*, 2009; Pires *et al.*, 2010; Pires *et al.*, 2011). The aim of this work was to compare GAS isolates from healthy carriers and patients with noninvasive and invasive infections regarding the presence and expression of selected virulence-related determinants. A total of 208 GAS [93 from oropharyngeal colonization (OC), 103 from noninvasive tonsillitis/pharyngitis (T/P; n=72) and skin/soft tissue infections (S/STI; n=31) and 12 invasive disease isolates (ID)] were typed by PFGE and *emm*-typing and screened for the presence of 12 virulence genes (*speA*, *speC*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *prtF1*, *spd1*, *slaA*, *ssa*). Expression of four genes (*speA*, *ssa*, *slaA* and *spd1)* was studied among 20 strains, chosen based on the association of same PFGE pattern/different clinical origins, using reverse transcriptase-PCR (RT-PCR) and cultures at late log phase in 2YT and BHI media. All genes were detected in OC isolates ranging from 5% for *speH* to 41% for *spd1*. However, all the genes were significantly (p<0.05) more frequent in ID isolates (min. 25% for *speA*, max. 83% for *speI*), except *ssa* that was infrequent in ID isolates (8%) and not in OC (32%). *speC*, *speM*, *speI* and *speL* were significantly (p<0.05) more frequent (30-80%) in noninvasive and ID isolates. The frequency of *speH* was significantly (p<0.05) higher in T/P (18%) and ID (25%). The *prtF1* gene was comparable in T/P (43%) and ID (41%) as well as in OC (30%) and S/STI (26%) isolates. *In vitro* expression of *ssa* was observed in a T/P isolate and not in OC and S/STI isolates. On the contrary, *spd1* was expressed in OC isolates and not in the S/STI isolate. *speA* and *slaA* were not expressed in any of the strains either from OC or infection origins. Several virulence genes were found in higher percentages among isolates from infection, particularly ID, than among colonization isolates. However, no consistent results were found regarding their expression, still raising the question of which factors may contribute to GAS infections.

Keywords: *Streptococcus pyogenes*, virulence factors, colonization, infection.

INTRODUCTION

Group A *Streptococcus* (GAS) (*Streptococcus pyogenes*) is among the most ubiquitous and versatile of human bacterial pathogens. It causes common clinical infections as pharyngitis, impetigo, cellulitis and scarlet fever. However, it may also cause acute and life-threatening diseases such as puerperal sepsis, necrotising fasciitis and streptococcal toxic shock syndrome (STSS) (Cunningham, 2000; Bisno *et al.*, 2003).

Over the past 20 years, there has been a significant increase in the incidence of invasive disease caused by *S. pyogenes* (Stevens *et al.*, 1989; Johnson *et al.*, 1992). Superantigens are believed to be important virulence factors of this pathogen (Commons *et al.*, 2008). They can induce massive secretion of inflammatory cytokines, such as gamma interferon, interleukin-1, and tumor necrosis factor-α. Overproduction of these cytokines can lead to tissue damage, organ failure, and shock (Chatellier *et al.*, 2000).

Currently, eight superantigen-encoding genes are known to be phage-encoded: *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM* (streptococcal pyrogenic exotoxins), and *ssa* (streptococcal superantigen) (Lintges *et al.*, 2010). Streptococcal phospholipase A_2 (*slaA*) and streptococcal phage DNase 1 (*spd1*) are also known as virulence factors associated to phages (Green *et al.*, 2005a). The *prtF1* gene that codifies to one fibronectin-binding protein (protein F1), is an important virulence factor because this surface protein is associated with adherence and invasion of epithelial cells (Bisno *et al.*, 2003).

In the present study, 208 *S. pyogenes* isolates from four different origins were analysed, such as oropharyngeal colonization, tonsillitis/pharyngitis, skin/soft tissue infections and invasive disease. Our aim was to observe if there are any differences in the distribution of virulence determinants, namely *speA*, *speC*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *ssa*, *prtF1*, *spd1* and *slaA* among the isolates of the different origins.

MATERIALS AND METHODS

Collection characterization

From a total of 1,541 isolates, 208 isolates were selected for this study. The 208 isolates were associated to four different clinical origins (98 from oropharyngeal colonization, 72 from tonsillitis/pharyngitis, 31 from skin/soft tissue infections and 12 from invasive disease).

The criteria for the selection of the 208 isolates were the following: same pulsed-field gel electrophoresis (PFGE) pattern/different origin; for each association PFGE pattern/different origin a maximum of two isolates sharing a same *emm*-type were chosen.

Media, bacterial growth and storage conditions

The growth medium generally used for the routine growth of cultures for colony isolation was Tryptic Soy Agar (TSA, Difco, Sparks, USA) supplemented with 5% sheep blood (Probiológica, Belas, Portugal). Cultures were incubated at 37ºC during 14-16 h and were stored at -80ºC in Brain Heart Infusion liquid medium (BHI, Biokar Diagnostics, Brookline, USA) with 20% glycerol.

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Presence or absence of virulence genes

DNA isolation

The strains stored at -80ºC were thawed and streaked onto plates of TSA with 5% sheep blood. They were incubated during 14-16 h at 37 ºC. After growth, cells were collected with a full loop and resuspended in 250 µl of TE (100mM Tris; 10mM EDTA) with lysozyme (10 mg/L) and incubated during 1 h at 37ºC. Total DNA was isolated following the guanidium thiocyanate method (Pitcher *et al.*, 1989).

PCR assays

In this study the presence of the following virulence genes was searched for: *speA, speC, speH, speJ, speI, speK, speL* and *speM, prtF1, spd1, slaA* and *ssa*, according to the description in Table 6.1.

The χ^2 analysis of contingency tables was used to determine the statistical independence between presence of virulence genes and origin of the isolates (Zar, 1996).

The boolean data matrix was introduced in BioNumerics® software (version 4.61; Applied Maths, Sint-Martens-Latem, Belgium) for construction of a dendrogram with the aim of selection of 20 isolates (10% of total collection) for gene expression analysis. Resemblance was computed with the simple matching coefficient and the unweighted pair-group method with arithmetic mean (UPGMA). The 20 isolates were selected using the following criteria:

resemblance level higher than 70%, different PFGE patterns but the most common among the total sample, higher quantity of genes and different origins.

Analysis of gene expression: preliminary studies

Growth assays

The growth assays were performed using the equipment Microbiology Workstation Bioscreen C® (ThermoLabSystems). This device monitors the optical density (OD) during the assay, generating growth curves and automatic calculation of growth parameters (e.g. generation time and maximum growth rate) (Carlos *et al.*, 2010).

The 20 selected strains were incubated at 37ºC during 14-16 h in 2YT medium [2× yeast tryptone – 1,6% (w/v) tryptone, 1,0% (w/v) yeast extract and 0,5% (w/v) NaCl] pH 7, in order to guarantee that cultures were in an active phase. Therefore, OD was measured for the collection of approximately 10^9 CFU (colony-forming units). The pellets obtained through centrifugation were resuspended in 100 µl of 10 mM phosphate buffer (PBS) pH 7. The 2YT medium, which is the control, and BHI medium, simulate infection conditions and were distributed in one Bioscreen C® microwell plate (each plate contains 100 wells and each well was filled with 300 µl of medium). Then, each well was inoculated with 3 µl of cell suspension. The plates were incubated into the equipment at 37ºC (24 h). OD readings were perfomed each 30 min, with prior agitation of 30 s. The wide band filter was used, which includes the wavelengths of 420 nm until 580 nm in order to minimize the influence of medium color changes during the assay (Carlos *et al.*, 2010). The assay was done in triplicate.

Collection of cells in specific points of the growth curve

After the Bioscreen C® assay, the number of hours that each isolate lasts to reach the stationary phase was determined for each culture medium. The following figure (Fig. 6.1) illustrates a typical bacterial growth curve, where the lag phase corresponds to adaptation of microrganisms to the medium, the exponential phase to a high duplication rate, the stationary phase to the reduction of nutrients and decline phase to the end of nutrients and consequent decrease of bacterial growth. The T1 and T2 points indicated in Fig. 6.1. correspond to the end of exponential phase and 2 h later, respectively, and represent the moments when culture samples were collected for further analysis.

So, initially the cultures grew during 16 h in 2YT medium, pH 7, at 37ºC, and ODs were measured as described above in order to the number of cells to be collected was identical (10^9 CFU). The cells were collected by centrifugation, washed with 500 µl of PBS and resuspended in 100 µl of the same buffer. Therefore, 10 ml of each of the selected media (2YT e BHI) was inoculated with 100 µl of bacterial suspension. The inocula grew at 37ºC, during the

number of hours determined for each isolate, in order to reach the T1 and T2 points, previously selected for cells collection.

Table 6.1. Primers and associated controls used in PCR reactions.

	Gene	Primer sequence (5'→ 3')	Amplicon size (bp)	Positive control	Reference
multiplex 1	*speA*	F:TAAGAACCAAGAGATGG R:ATTCTTGAGCAGTTACC	248	S69*	Schmitz *et al.*, 2003
	speC	F:GATTTCTACTTATTTCACC R:AAATATCTGATCTAGTCCC	584	S69*	
	speH	F:AGATTGGATATCACAGG R:CTATTCTCTCGTTATTGG	416	1002*	
	speJ	F:ATCTTTCATGGGTACG R:TTTCATGTTTATTGCC	535	S69*	
multiplex 2	*speI*	F:AAGGAAAAAATAAATGAAGGTCCGCCAT R:TCGCTTAAAGTAATACCTCCATATGAATTCTTT	217	GAP 65[§]	Lintges *et al.*, 2007
	speM	F:CCAATATGAAGATAACAAAGAAAATTGGCACCC R:CAAAGTGACTTACTTTACTCATATCAATCGTTTC	600	VSD7[§]	
multiplex 3	*speK*	F:TACAAATGATGTTAGAAATCCAAGGAACATATATGCT R: CAAAGTGACTTACTTTACTCATATCAATCGTTTC	656	VSD1[§]	Lintges *et al.*, 2007
	speL	F:GGACGCAAGTTATTATGGATGCTCA R:TTAAATAAGTCAGCACCTTCCTCTTTCTC	460	GAP 17[§]	
	prtF1	F:TATCAAAATCTTCTAAGTGCTGAG R:AATGGAACACTAACTTCGGACGGG	780 - 1200	DSMZ2071[£]	Talay *et al.*, 1994
	spd1	F:CCCTTCAGGATTGCTGTCAT R:ACTGTTGACGCAGCTAGGG	400	GAP 17[§]	Green *et al.*, 2005a
	slaA	F:CTCTAATAGCATCGGCTACGA R:AATGGAAAATGGCACTGAAAG	440	GAP 106[§]	Green *et al.*, 2005a
	ssa	F:AGTCAGCCTGACCCTAC R:TAAGGTGAACCTCTAT	691	8563*	Reda *et al.,* 1994
	rRNA16S	F - AGAGTTTGATCCTGGCTCAG R - CCGTCAATTCMTTTRAGTTT	907		Massol-Deya *et al.,*1995

Legend: * *S. pyogenes* strain provided by Birgitta Henriques-Normark; [§] Bovine *Streptococcus dysgalactiae*, subsp. *dysgalactiae* (VSD1, VSD7) and *S. pyogenes* (GAP 65, GAP 17, GAP 106) strains from our laboratory collection (Head: Ilda Santos-Sanches); [£] Reference strain from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Alemanha).

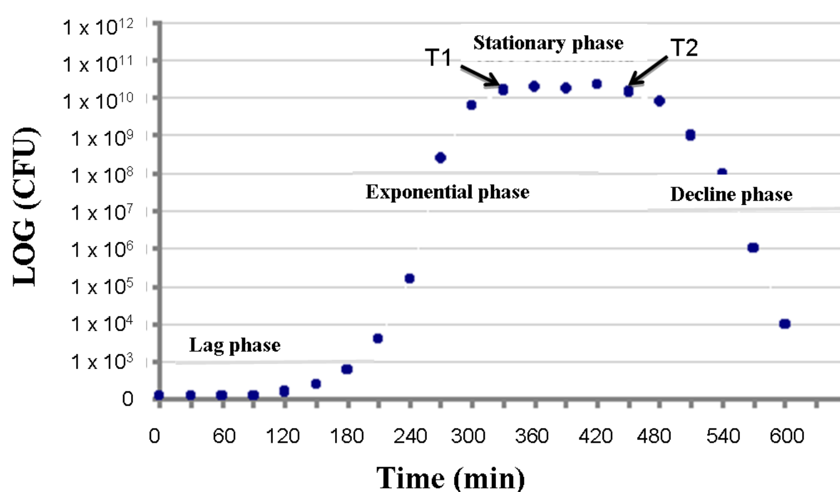

Figure 6.1. Typical growth pattern of a microbial culture.

Legend: T1 and T2 correspond to the two moments of cell collection. T1 represents the end of exponential phase and corresponds to the first collection point (approximately 330 min in this graphic) and T2 correspond to the second point, which is 2h after T1.

RNA isolation

In T1 and T2 points, cells were collected by centrifugation and resuspended in 1 ml of TE. RNA isolation was performed using the Trizol® (Invitrogen, Life Technologies, Carlsbad, USA) method. For confirmation of RNA quality/quantity, 5 µl of the sample was observed after electrophoresis in 1% of agarose in 0,5X TBE buffer at a voltage of 95V during 90 min. Therefore, it was performed the treatment with DNase I (Invitrogen, Life Technologies, Carlsbad, USA), in order to remove residual DNA. Each 1 µg of RNA was treated with 1 U of DNase I and incubated at 37ºC during 45 min. Then, 1 µl of 25 mM EDTA was added and incubated at 65ºC during 15 min (Carlos *et al.*, 2010). The efficiency of the treatment was confirmed by amplification of rRNA 16S housekeeping gene. The absence of amplification among RNA samples and amplification among DNA sample (positive control) confirmed the efficiency of the treatment.

Reverse transcriptase PCR (RT-PCR) technique

For the synthesis of cDNA, 100 ng of total RNA treated with DNase was added, as well as 1 µl of 300 ng/µl random primers, 1 µl of 10 mM deoxynucleotide triphosphates (dNTPs) and sterile water treated with diethylpyrocarbonate (DEPC) to a final volume of 13 µl. The mixture was heated at 65ºC during 5 min and rapidly incubated on ice during 1 min. Therefore, 4 µl of 5X First-Strand Buffer, 1,5 µl of 0,1 M dithiothreitol (DTT), 1 µl of 40 U/µl RNaseOUT™ Recombinant RNase Inhibitor and 0,5 µl of 200 U/µl SuperScript™ III RT were added (final

volume 20 µl). The solution was mixed, kept at 25ºC during 10 min, incubated at 50ºC for 1 h and heated for enzyme inactivation at 70ºC during 15 min (Carlos *et al.*, 2010).

PCR assay

The cDNAs obtained were used for amplification of *speA*, *ssa*, *slaA* and *spd1* genes. The rRNA 16S housekeeping gene was used as control. The mix was prepared with the following reagents: 2,5 µl of 10X PCR buffer, 1,5 µl of 50 mM $MgCl_2$, 0,5 µl of 10 mM dNTPs, 0,5 µmol of each primer, 0,2 µl of 5 U/µl Taq DNA polymerase and 1 µl of cDNA. PCR reactions were performed as described in Table 6.2. For confirmation of PCR amplification, 5 µl of the sample was observed after electrophoresis in 1% of agarose gel in 0,5X TBE buffer at a voltage of 95V during 90 min.

Data analysis

The captured gel images were analyzed by software ImageJ 1.40g (National Institute of Health, USA). The integrated density was calculated through the selection of the entire area of each amplicon. To compare the influence of different media on the expression of virulence genes, each integrated density value was normalized to the housekeeping gene rRNA 16S, according to equation 1, to obtain its corresponding expression level (EL) (Carlos *et al.,* 2010):

$$EL = \frac{\text{Virulence gene x in the culture medium A}}{\text{Housekeeping gene x in the culture medium A}}$$

Table 6.2. Primers used in PCR reactions.

Gene	Sequence of primer (5'→ 3')	Length of amplicon	Reference
speA	F: 5' TAA GAA CCA AGA GAT GG R: 5' ATT CTT GAG CAG TTA CC	248 bp	Schmitz *et al.*, 2003
ssa	F: 5' AGT CAG CCT GAC CCT AC R: 5' TAA GGT GAA CCT CTA T	691 bp	Reda *et al.*, 1994
slaA	F: 5'CTC TAA TAG CAT CGG CTA CGA R: 5' AAT GGA AAA TGG CAC TGA AAG	440 bp	Green *et al.*, 2005a
spd1	F:5' CCC TTC AGG ATT GCT GTC AT R: 5' ACT GTT GAC GCA GCT AGG G	400 bp	Green *et al.*, 2005a

RESULTS

Presence or absence of virulence genes

A total of 12 virulence genes (*speA*, *speC*, *speH*, *speJ*, *speI*, *speK*, *speL*, *speM*, *prtF1*, *spd1*, *slaA* and *ssa*) were searched for among 208 selected isolates from four different origins: oropharyngeal colonization (93 isolates), tonsillitis/pharyngitis (72 isolates), skin/soft tissue infections (31 isolates) and invasive disease (12 isolates). This analysis was performed in order to found if there were significant differences related with the genes detected among isolates from different origins. Figure 6.2. indicates the presence of virulence genes by origin.

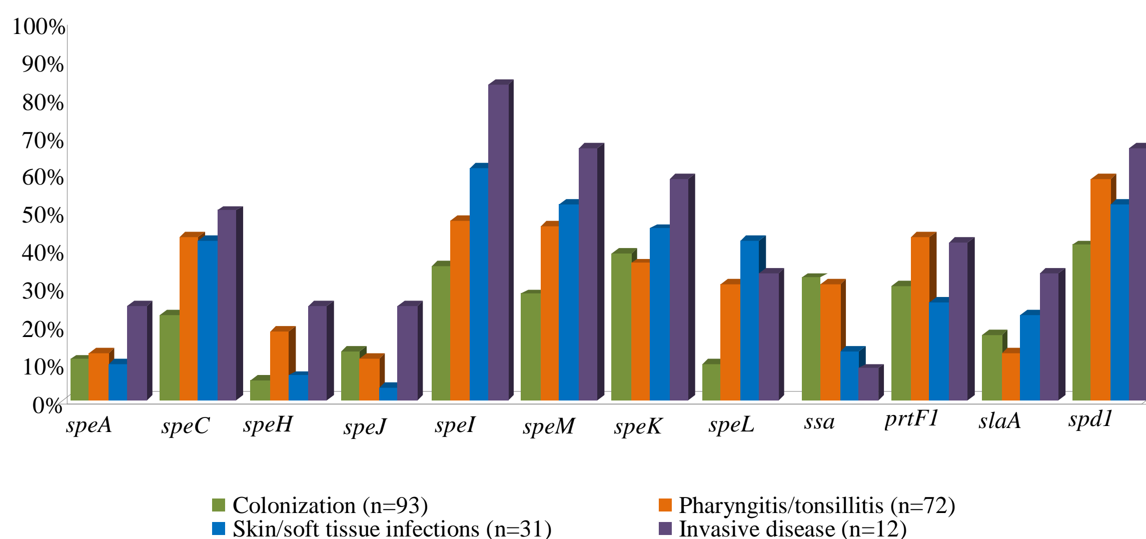

Figure 6.2. Presence of virulence genes among different origins.

The percentages are related to the total number of isolates by each origin included in this study.

As we can observe in Fig. 6.2, for the majority of genes invasive disease presents a higher incidence, in contrast with colonization isolates which, with the exception of *ssa* (32%), always presented low percentages. Percentages of presence of virulence genes varied in colonization between 5% (*speH*) to 41% (*spd1*), in tonsillitis/pharyngitis between 11% (*speJ*) to 58% (*spd1*), in skin/soft tissue infections between 3% (*speJ*) and 62% (*speI*) and in invasive disease among 8% (*ssa*) to 83% (*speI*).

Relatively to *spe* genes, all but *speL*, *slaA* and *spd1* were found as more common among isolates from invasive disease, comparing with isolates of the other three origins. Among the isolates from colonization, the percentages were the lowest among five of the eight *spe* genes.

The *prtF1* gene was found in similar percentages among isolates of two origins: tonsillitis/pharyngitis (43%) and invasive disease (41%). The percentages for the other two origins were also similar (about 26%).

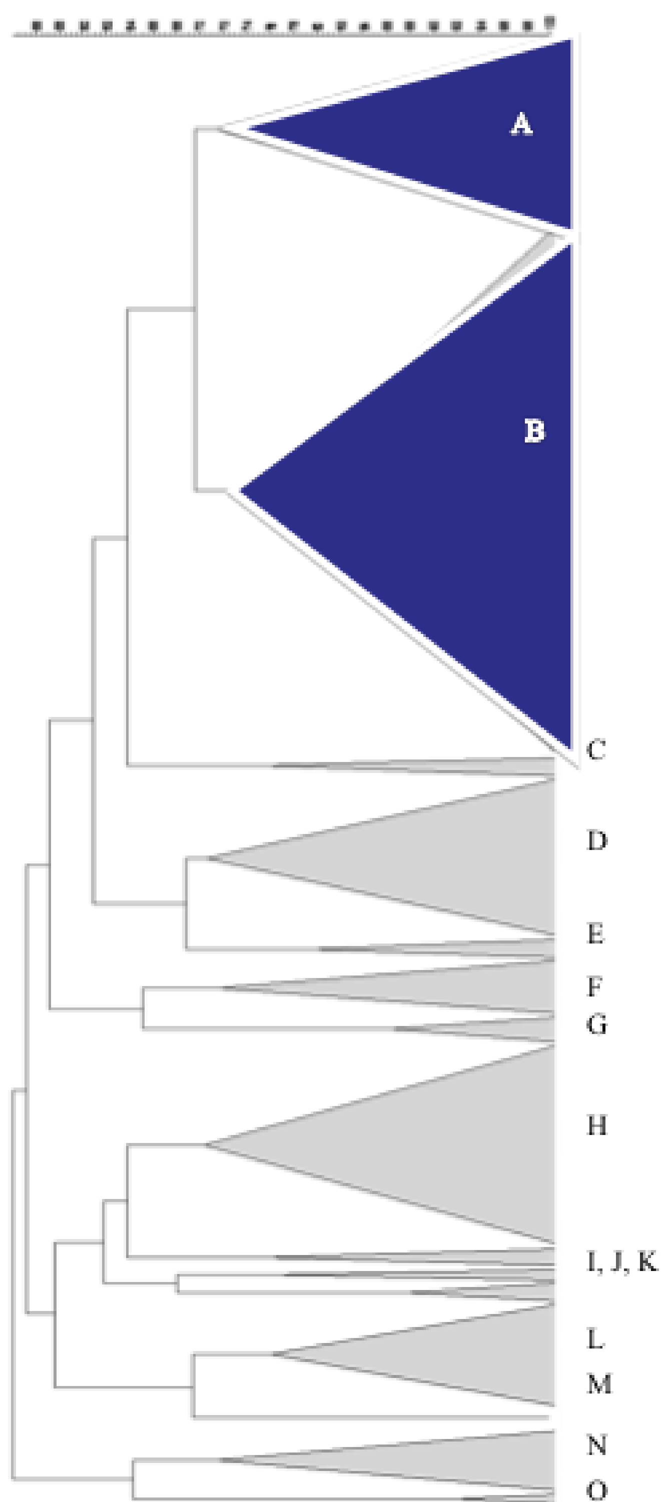

Figure 6.3.A. Representative dendrogram of virulence genes searched for among 208 GAS isolates.

Resemblance was calculated with the simple matching coefficient and agglomerative method UPGMA.

Cluster A (1 to 4 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
17	7	3	-	27

Cluster B (1 to 3 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
41	21	12	2	76

Cluster C (4 to 5 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
1	2	-	-	3

Cluster D (2 to 7 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
11	9	3	-	23

Cluster E (4 to 6 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
1	2	-	-	3

Cluster F (4 to 6 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
6	2	-	-	8

Cluster G (4 to 5 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
-	3	-	1	4

Cluster H (4 to 9 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
3	16	7	3	29

Cluster I (4 to 6 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
-	2	1	-	3

Cluster J (5 to 6 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
1	1	-	-	2

Cluster K (3 to 5 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
1	1	1	-	3

Cluster L (4 to 7 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
7	3	2	3	15

Cluster M (6 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
-	-	-	1	1

Cluster N (3 to 7 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
2	3	2	2	9

Cluster O (6 to 7 virulence genes/isolate)

Colonization	Tonsillitis/pharyngitis	Skin/soft tissue	Invasive disease	Total
2	-	-	-	2

Figure 6.3.B. Distribution by clinical origin of the 208 GAS isolates of clusters A-O.

Among each cluster is indicated the number of associated isolates and their distribution among the four origins.

The *slaA* and *spd1*, both phage-encoded genes, were distributed in higher percentages among isolates from invasive disease (33% and 66%, respectively), comparing with isolates of other origins.

The χ^2 analysis applied to the presence/absence of virulence genes revealed that *speC*, *speH*, *speI*, *speM* and *speL* presented statistically significant differences ($p<0.05$) relatively to their origins. The statistical analysis showed that the same virulence genes presented statistically significant differences ($p<0.05$) taking into account the origin colonization, and *speH* gene also presented statistically significant differences to the same origin, as well as to the origin skin/soft tissue infections. Relatively to the remaining virulence genes, it was not observed any statistically significant difference when their presence was analyzed among the isolates of all origins.

For a more detailed analysis of genes distribution among the four origins, one table of boolean data was introduced in BioNumerics® software for the construction of a dendrogram (Fig. 6.3.). Considering a resemblance of 70%, we can observe 15 clusters represented by letters (A-O). With this dendrogram, and taking into account resemblance values higher than 70%, it was possible to select 20 strains for the gene expression study using the following criteria: different PFGE types but the most common among the collection, higher quantity of genes and different origins.

With the analysis of the dendrogram, clusters A and B represented about 50% of the total sample of isolates. Among these two clusters, the quantity of virulence genes by isolate varied between zero and four, whereas in the remaining clusters varied between two and nine. Clusters A and B contained about 62% of the isolates from colonization, 38% from tonsillitis/pharyngitis, 48% from skin/soft tissue infections and 16% of invasive disease. These results could confirm that the isolates from colonization contain less virulence genes comparing with the isolates from the remaining origins.

The most frequent *emm*-types found were *emm*4 (12%), *emm*1 (9%) and *emm*28 (7%). The virulence genes found in more than a half of the *emm*4 isolates isolates were *ssa* (88%), *spd1* (67%) and *prtF1* (54%). Among the *emm*1 isolates, the 12 virulence genes were present in less than 50% of the isolates. Among *emm*28 isolates, the most common virulence genes found were *spd1* (100%), *prtF1* (79%), *speC* (64%) and *speI* (57%).

Besides these *emm*-types referred to above, 20 other *emm*-types already known were identified among the 208 isolates. One new *emm*-subtype 53.10 was found (GenBank accession number FJ711063).

Gene expression analysis: preliminary studies

The growth assays at Microbiology Workstation Bioscreen C® generated 60 growth curves (3 curves by isolate) for the control medium (2YT) and for the medium used to simulate infection conditions (BHI). To determine T1 and T2 for cells collection, the average of the three growth curves for each isolate and culture medium was calculated. The results are showed in Table 6.3.

Table 6.3. Average hours for the collection of cells of 20 selected strains for gene expression analysis.

Isolate	2YT		BHI	
	T1(h)	T2(h)	T1(h)	T2(h)
GAP 38	6:00	8:00	6:00	8:00
GAP 83	6:00	8:00	7:00	9:00
GAP 106	7:30	9:30	8:30	10:30
GAP 111	4:42	6:42	6:00	8:00
GAP 115	5:30	7:30	6:00	8:00
GAP 124	6:00	8:00	5:18	7:18
GAP 132	4:00	6:00	6:00	8:00
GAP 152	5:00	7:00	5:18	7:18
GAP 174	4:00	6:00	4:30	6:30
GAP 279	5:00	7:00	7:00	9:00
GAP 308	6:00	8:00	7:48	9:48
GAP 426	4:30	6:30	5:30	7:30
GAP 511	4:12	6:12	6:00	8:00
GAP 679	6:42	8:42	6:00	8:00
GAP 711	7:48	9:48	6:00	8:00
GAP 801	7:00	9:00	7:18	9:18
GAP 823	5:12	7:12	6:00	8:00
GAP 847	4:30	6:30	3:42	5:42
GAP 1006	7:00	9:00	5:30	7:30
GAP 1106	6:42	8:42	5:30	7:30

By analysis of Table 6.3. and by the observation of the graphics presented in Figure 6.4., the isolates were maintained during more time in exponential phase and reached higher ODs in BHI culture medium as expected because this medium has more nutrients.

The gene expression analysis was performed for the 20 selected strains using the following genes: *speA*, *ssa*, *slaA* and *spd1*. We only observed expression for *ssa* and *spd1* genes, demonstrating that *speA* and *slaA* genes are not being expressed in the analyzed culture media and /or during the selected times of collection.

The Figure 6.5 shows the results of gene expression analysis. Out of nine isolates that presented expression, four were from colonization, three were from skin/soft tissue infections and two were from tonsillitis/pharyngitis and invasive disease. The expression levels higher than the observed for the housekeeping gene rRNA 16S were found only among two isolates: *spd1* in one colonization isolate; *ssa* in one tonsillitis/pharyngitis isolate. The same EL was found for *ssa* in one skin/soft tissue infection isolate. For the remaining isolates, where it was observed a lower expression level of *spd1* and *ssa* comparing with the housekeeping gene (EL<1), the genes analyzed were sub-expressed in comparison with rRNA 16S. *speA* and *slaA* were not expressed in any of the strains either from colonization or infection origin.

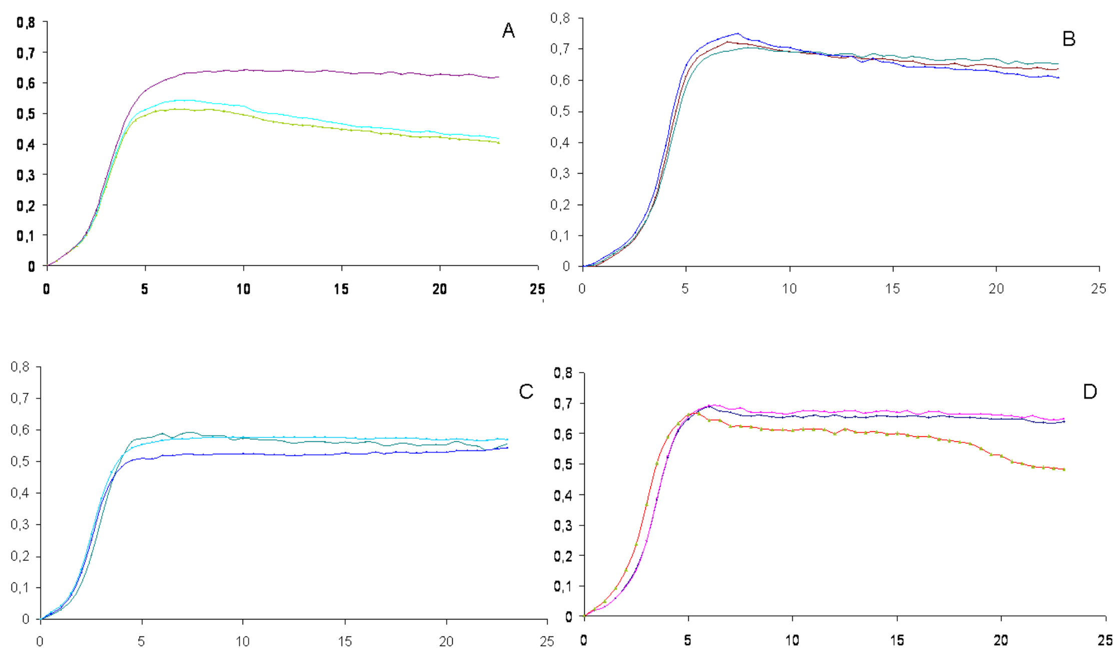

Figure 6.4. Growth curves in 2YT and BHI media.

The x axe represents the time (h) and y axe represents the OD. Isolate GAP 111 (from invasive disease) in 2YT (A) and BHI (B) culture media. Isolate GAP 426 (from skin/soft tissue infection) in 2YT (C) and BHI (D) culture media. Each graphic presents three replicates by isolate.

DISCUSSION

In the present study, the searching of virulence genes among *S. pyogenes* isolated from four different origins was performed in order to verify if there were differences in the presence of the referred genes among the origin of the isolate. After analysis of the results, we concluded that virulence genes are found in higher percentages among isolates from disease, mostly from invasive disease. Previous studies from other countries, where search of virulence genes was also performed, have similar conclusions (McMillan *et al.*, 2006; Rivera *et al.*, 2006; Commons *et al.*, 2008; Maripuu *et al.*, 2008).

Despite the study of searching for virulence genes is necessary, it becames insufficient, being important to complete it with gene expression analysis. In the present study, such analysis was not conclusive because it was not possible to search for the 12 virulence genes among the total number of isolates, and also because *S. pyogenes* is a β-hemolytic species and is expected that it express more virulence genes when it is found in infection situations, so it would be mandatory the usage of a culture medium which simulates infection conditions, such as animal serum.

In the future, with the purpose of obtaining better results, the reverse transcriptase method should be changed by real-time PCR technique or, ideally, by microarrays. Real-time PCR is a quantitative PCR that can be used to analyze isolates from colonization and infection, giving information about expression in different media, in real time, having the advantage of being a less expensive technique. On the other hand, the expression analysis using microarrays technique for bacteria cultured *in vivo* seems to be a good choice to verify gene expression adjusted to all factors involved, because the current knowledge about bacterial gene expression is based mostly in studies performed under controlled lab conditions, where typically only one variable is tested each time. Despite this approach contributes for virulence studies, it is not able to simulate all host factors where the pathogen develops (Beyer-Sehlmeyer *et al.*, 2005; Graham *et al.*, 2006; Rosey *et al.*, 2007; Lee *et al.*, 2008; Curran *et al.*, 2010).

The genetic properties of *S. pyogenes* strains vary according the geographic region, being also important the role of population density and climatic conditions. In Portugal, there was not any study on the presence of all virulence genes included in this study. So, this work contributes to a better knowledge of *S. pyogenes* virulence factors in this country, and completed previous studies and could relate the searched virulence genes with other aspects, such as resistance factors and *emm*-type.

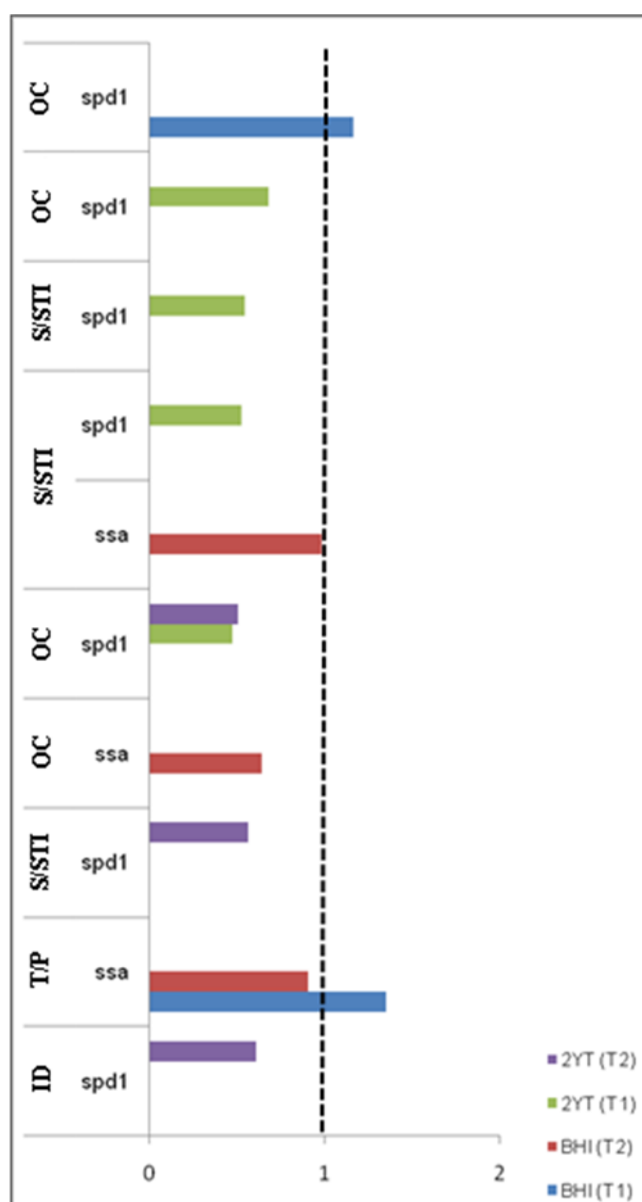

Figure 6.5. Graphic showing the expression levels for the selected strains.

Only the isolates with gene expression in 2YT and BHI media, for T1 and T2 times, were represented. The dashed line represents expression level equal to the housekeeping gene 16S rRNA. OC – oropharyngeal colonization; S/STI – skin/soft tissue infection; T/P – tonsillitis/pharyngitis; ID – invasive disease.

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All the experimental work was performed by R. Pires, with the exception of the identification and characterization of part of the isolates which was carried out with the collaboration of Lara Lino and other graduate and undergraduate students of the laboratory

Chapter VII

CONCLUDING REMARKS

S. pyogenes is considered to be the most pathogenic bacterium in the genus *Streptococcus*. The reason why this microorganism is a major public health concern is because is one of the most versatile and common human pathogens, causing a wide spectrum of diseases, ranging from mild infections to life-threatening systemic diseases (Facklam, 2002). Although it not be considered normal flora, *S. pyogenes* can colonize oropharyngeal respiratory tract without manifestation of clinical infection symptoms by the host (Cunningham, 2000). Colonization is also considered an infection; however the association between the microorganism and the host is commensal (Berkovitch *et al.*, 2002).

At the beginning of these doctoral studies, in 2007, some of the fundamental questions concerning the antimicrobial susceptibility, molecular epidemiology and virulence profiling of *S. pyogenes* colonization isolates remained unanswered. On the other hand, reports on the comparison of genotypes of isolates from symptomatic and asymptomatic infections were scarce.

The work presented in this Thesis shed light into these two subjects: the molecular characterization of isolates from oropharyngeal colonization and their comparison with isolates from symptomatic infections.

Insights into the carrier state

The *S. pyogenes* carrier is an asymptomatic individual with a positive oropharyngeal swab culture and without serological response, or with a positive culture after completing the appropriate treatment with antimicrobial agents (Martin *et al.*, 2004). The carrier state may be clinically important because colonization may provide useful information about the prevalent phenotypes and clones within the community, as they may be present among infections (Durmaz *et al.*, 2003; Fazeli *et al.*, 2003; Hoe *et al.*, 2003; Pires *et al.*, 2005).

In order to evaluate the trends of oropharyngeal colonization, in Chapter II, a collection of *S. pyogenes* isolates from several DCCs and schools was fully characterized in terms of their PFGE patterns and *emm*-types.

Before this study, the *S. pyogenes* carrier state in Portugal was not known. The results presented in Chapter II of this Thesis provided evidence that the mean carrier rate among pre-school children (0-6 years) was higher (11.5%) than among school-aged children (7-16 years) (7.8%) and that colonization was higher during winter periods, which suggests that the crowding of children in DCCs may possibly increase the carrier rate in healthy pre-school children. It was demonstrated that a very heterogeneous population of *S. pyogenes* colonized healthy carriers. Moreover, it was also found that a high diversity of *S. pyogenes* strains was associated with long-term colonization. Also, a replacement of *S. pyogenes* strains was

frequently found among recurrent carriers and co-colonization of the oropharynx by *S. pyogenes* strains was detected.

Future analyses will be needed for the comparison of major and minor clones that are able to cause asymptomatic colonization, e. g. the determination of virulence profiles in these two sets of isolates.

Bacitracin resistance in *S. pyogenes* from colonization and disease

One of the presumptive tests for *S. pyogenes* identification is the susceptibility to bacitracin, which provides the differentiation from other beta-hemolytic streptococci of human origin (Facklam, 2002). However, resistance to this antimicrobial agent in *S. pyogenes* was previously described (Facklam and Washington II, 1991), raising questions concerning the reliability of this criterion. Resistance to bacitracin in *S. pyogenes* has been also frequently associated to constitutive resistance to macrolides, lincosamides and streptogramins B ($cMLS_B$ phenotype) (Malhotra-Kumar *et al.,* 2003; Mihaila-Amrouche *et al.,* 2004; Pérez-Trallero *et al.,* 2004; Pires *et al.*, 2009).

In order to assess the putative variability of genetic backgrounds, as well as to evaluate the bacitracin resistance mechanism in *S. pyogenes* from colonization and disease in 2000-2007, a collection of 45 *S. pyogenes* isolates was fully characterized.

According to literature, no data was previously published regarding bacitracin resistance among *S. pyogenes* colonization isolates. In this work (see Chapter V), we reported the long-term persistence among carriers of the low-level bacitracin-resistant *emm*28/ST52 lineage, which is prevalent in Europe. We also reported for the first time a high-level bacitracin-resistant isolate of the *emm*74/ST120 lineage, which was not previously known to include bacitracin-resistant isolates. However, bacitracin resistance mechanism(s) in those isolates remain unknown, so further investigation will contribute to ascertain the genetic basis for bacitracin resistance. It would be interesting to test if *bacA* gene is overexpressed in *emm*28/ST52 and *emm*74/ST120 lineages and/or if the ABC transporter encoded by *mbrABCD* genes in *S. mutans* (Tsuda *et al.*, 2002) is present among these two lineages.

Resistance to macrolides among healthy children

S. pyogenes is uniformly susceptible to penicillin and this is the universal recommended treatment for *S. pyogenes* infections. However, macrolides represent an alternative in patients who are allergic to penicillin (Bisno *et al.*, 2002), and consequently increased frequency of macrolide-resistant *S. pyogenes* from infection sites has been reported in different countries (Felmingham *et al.*, 2004) including from asymptomatic oropharyngeal colonization (Creti *et*

al., 2005; Pires *et al.*, 2005; Chang *et al.*, 2010). In Portugal, it was unknown the role of healthy carriers as disseminators of macrolide-resistant *S. pyogenes* isolates, in contrast with patients with tonsillo-pharyngitis (Silva-Costa *et al.*, 2006; Silva-Costa *et al.*, 2008).

Chapter III described the frequency of macrolide-resistant *S. pyogenes* recovered in Lisbon area, Portugal, from healthy children during 2000 to 2006, as well as the capacity of asymptomatic carriers as reservoirs of macrolide-resistant and potential virulent clones capable of causing a wide spectrum of infections.

Temporal inversions of macrolide resistance phenotypes among colonization isolates were first reported and described in Chapter III, reinforcing the importance of surveillance of carriers, as they may be indicators of the pool of isolates circulating in the community that may cause infections. The elevated frequency of macrolide resistance was associated with M phenotype lineages *emm*12/ST36 and *emm*4/ST39, and with one $cMLS_B$ lineage *emm*28/ST52, known as associated with upper respiratory tract and invasive infections. The $cMLS_B$ lineage *emm*11/ST403 was found in this study for the first time among colonization isolates. The high prevalence (>20%) of virulence genes *speC*, *prtF1* and *ssa* was probably caused either by clonal dissemination (*speC*), or to horizontal gene transfer events (*prtF1* and *ssa*). This study contributed to a better understanding of the molecular epidemiology and evolution of macrolide-resistant *S. pyogenes* causing asymptomatic oropharyngeal colonization. These colonizing strains carry macrolide resistance and virulence genes capable of being transferred to other bacterial species sharing the same niche.

Ciprofloxacin nonsusceptibility in healthy children and pediatric patients

The widely use of fluoroquinolones has led to the emergence of *S. pyogenes* isolates with reduced susceptibility to these antimicrobial agents (Orscheln *et al.*, 2005; Yan *et al.*, 2008; Smeesters *et al.*, 2009; Montes *et al.*, 2010) or with high-level resistance (Yan *et al.*, 2000; Malhotra-Kumar *et al.*, 2009; Montes *et al.*, 2010). Nonsusceptibility to fluoroquinolones in *S. pyogenes* from infections has been reported worldwide, ranging from 3.2% in Spain (Rivera *et al.*, 2005) to 22.5% in Belgium (Smeesters *et al.*, 2009), however few surveys included isolates exclusively derived from pediatric patient populations (Albertí *et al.*, 2005; Yan *et al.*, 2008; Smeesters *et al.*, 2009). Moreover, to the best of our knowledge, there are no reports documenting the prevalence and characterization of fluoroquinolone-nonsusceptible *S. pyogenes* from asymptomatic colonization.

In Chapter IV, the prevalence of ciprofloxacin-nonsusceptible *S. pyogenes* from colonized and infected Portuguese children from 1999 to 2006 was described and the associated clones and resistance mechanisms were characterized.

The data presented in Chapter IV demonstrated that ciprofloxacin nonsusceptibility rate was slightly lower among colonization isolates (4.3%) than among the clinical isolates (6.0%). The ciprofloxacin MIC≥2 µg/mL was a valuable breakpoint for the detection of 66 ciprofloxacin-nonsusceptible isolates; all but one ciprofloxacin-nonsusceptible isolates had *parC*-QRDR mutations generating the aminoacid substitutions S79A (n=63) and D83G (n=2); the ParC-D83G substitution was found for the first time in this study and *emm*1 association with fluoroquinolone-nonsusceptibility was also first detected in this work. One isolate (*emm*89/ST101) had no *parC*-, *parE*-, *gyrA*- and *gyrB*-QRDRs codon substitutions, nor plasmid-encoded *qnrA*, *qnrB*, *qnrC* and *aac(6')-Ib-cr* genes, nor reserpine-sensitive efflux pump. In this isolate, it would be interesting the searching for the presence of a fluoroquinolone efflux pump using other efflux pump inhibitors, e. g. thioridazine and verapamil.

In this study it was demonstrated that in Portugal fluoroquinolone resistance in pediatric *S. pyogenes* is mediated by both clonal dissemination and by the putative occurrence of horizontal gene transfer involving the *parC*-QRDR region.

Virulence factors in *S. pyogenes* from colonization and disease

Currently, eight superantigen-encoding genes are known to be phage-encoded: *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM* (streptococcal pyrogenic exotoxins), and *ssa* (streptococcal superantigen) (Lintges *et al.*, 2010). Streptococcal phospholipase A_2 (*slaA*) and streptococcal phage DNase 1 (*spd1*) are also known as virulence factors associated to phages (Green *et al.*, 2005b). The *prtF1* gene that codifies to one fibronectin-binding protein (protein F1), is an important virulence factor because this surface protein is associated with adherence and invasion of epithelial cells (Bisno *et al.*, 2003).

In order to know if the presence of streptococcal virulence factors is associated with the origin of the isolates, we performed a screening for the presence of virulence genes (*speA, speC, speH, speI, speJ, speK, speL, speM, prtF1, spd1, slaA, ssa*) in 208 isolates of *S. pyogenes* from four distinct origins: oropharyngeal colonization (OC), tonsillitis/pharyngitis (T/P), skin/soft tissues infections (S/STI) and invasive disease (ID). In order to complement the previous analysis, we selected four virulence genes (*speA*, *ssa*, *slaA* and *spd1*) and studied their gene expression in 20 isolates selected from the initial sample, after growth in two media, BHI and 2YT.

In Chapter VI, after the analysis of the results, we concluded that virulence genes are found in higher percentages among isolates from disease, such as T/P, S/STI or ID. In the present study, gene expression analysis was not conclusive because it was not possible to search for the 12 virulence genes among the total number of isolates, and it would be useful the usage of a culture medium which simulates infection conditions, such as animal serum. In Portugal,

there was not any study on the presence of all virulence genes included in this study. So, this work contributed to a better knowledge of *S. pyogenes* virulence factors in this country. In the future, the phage-encoded virulence genes included in this study will be used for a primary detection of prophages and to evaluate the polylysogenic nature of both colonizing and infection strains. So, lytic induction of prophages will be carried out after treatment with mitomycin C.

In conclusion, the work presented in this Thesis provided new insights into antimicrobial susceptibility, molecular epidemiology and virulence profiling of *S. pyogenes* isolates from oropharyngeal colonization and symptomatic infections. As it was described above, isolates from colonization and infection can share the same genetic backgrounds (i.e. the same PFGE pattern and/or *emm*-type), however infection isolates presented higher percentages of virulence genes, which could have an impact in their pathogenesis. Nevertheless, some questions arose from this work that remain to be clarified and would constitute an interesting subject for future investigations.

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