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Decrease in macrolide resistance and clonal instability among *Streptococcus pyogenes* in Portugal

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ABSTRACT

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

Keywords Clonal types, macrolide resistance, Portugal, resistance prevalence, sequence types, *Streptococcus pyogenes*

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INTRODUCTION

Streptococcus pyogenes, Lancefield group A streptococci (GAS), is an important human pathogen causing a wide variety of infections, from severe life-threatening diseases to pharyngitis, an infection for which it is the most common bacterial aetiological agent. β-Lactams remain the antibiotics of choice in the treatment of GAS pharyngitis, and macrolides and lincosamides are the first-line alternatives. Although macrolide resistance in GAS remained at low levels for a long time, a number of recent studies have reported an increase in resistance. Two different mechanisms have been recognized in macrolide-resistant *S. pyogenes*: target site modification and active efflux. Target site modification occurs in the ribosome via an erythromycin resistance methylase (Erm) protein, blocking the binding of macrolides, lincosamides and streptogramin B (generating the MLS_B phenotype) [1]. In GAS, the MLS_B phenotype can be mediated by two classes of methylase genes, the *erm*(B) determinant and the *erm*(TR) determinant (belonging to the *erm*(A) class) [2]. The expression of the *erm* genes can be either constitutive or inducible, generating the $cMLS_B$ phenotype or the $iMLS_B$ phenotype, respectively, the latter frequently being associated with the *erm*(A) class. Both classes of *erm* genes were found to be associated with transposons that were shown to have the capacity to transfer the

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resistance traits to susceptible isolates by conjugation [3]. The second mechanism conferring macrolide resistance in GAS is mediated by a membrane-associated pump encoded by the *mef* genes, leading to resistance to 14- and 15-membered ring macrolides (generating the M phenotype) [4]. The *mef*(A) and *mef*(E) variants are widely distributed in streptococci [5], although the *mef*(A) variant associated with a phage-like element was found in the majority of GAS with the M phenotype [6]. Other mechanisms of resistance resulting from mutations, such as alterations of the ribosomal proteins, are infrequently observed in isolates responsible for infections, and currently have little clinical impact [7].

The factor most frequently associated with increases in antimicrobial resistance is antimicrobial consumption [8,9]. An association between macrolide consumption and resistance in GAS was shown in ecological studies [9,10], with intermediate-acting (e.g. clarithromycin) and, particularly, long-acting (e.g. azithromycin) macrolides being implicated in enhanced resistance selection [11]. Further supporting this association, it was noted that a sharp decrease in macrolide prescribing was accompanied by a decline in macrolide-resistant GAS [12]. More recently, studies at the individual level confirmed and extended these findings by showing a causal relationship between both clarithromycin and azithromycin treatment and selection for macrolide-resistant streptococci [13]. Furthermore, the latter study implicated clarithromycin, but not azithromycin, in the selection for the *erm*(B) gene. In spite of the recognized importance of antibiotic consumption in the selection of resistant strains, the largely clonal structure of most bacterial populations [14], including GAS [15,16], suggests that the circulating clones may also contribute significantly to both the prevalence of resistance phenotypes and the overall level of resistance. The transmissibility of the genetic elements carrying the resistance determinants may also influence their prevalence in the population, with the more easily disseminated elements having an advantage over less mobile genetic determinants [17].

We have previously shown that, although erythromycin resistance in GAS remained above 20% in Portugal from 1998 to 2003, this was not associated with a stable population of macrolide-resistant *S. pyogenes* [18]. Indeed, the predominance of the MLS_B phenotype, which accounted for *c.* 80% of isolates in 1998, was completely reversed in 2003, when almost 77% of the isolates expressed the M phenotype [18]. We have also found these changes to be associated with the decline of a particular clone expressing the MLS_B phenotype and the emergence of several clones expressing the M phenotype [16]. Here we report the continuing fluctuations in macrolide resistance phenotypes and a decline in overall erythromycin resistance.

MATERIALS AND METHODS

Bacterial isolates and identification

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

Antimicrobial susceptibility testing and macrolide resistance phenotype

Susceptibility to erythromycin, clindamycin and tetracycline (Oxoid, Basingstoke, UK) was tested using disk diffusion according to CLSI recommendations [19]. The macrolide resistance phenotype was determined according to a double-disk test previously described [20].

Bacitracin susceptibility was determined for all isolates by disk diffusion using disks containing 0.05 U of bacitracin (Oxoid, Basingstoke, UK) as previously described [16]. The absence of an inhibition zone around the disk was interpreted as resistance.

PCR determination of the macrolide and tetracycline resistance genotype

Total bacterial DNA was isolated according to the methodology described by the CDC (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). PCR reactions to determine which of the macrolide resistance determinants (*erm*(B), *erm*(A) or *mef*) was present were performed as described previously [21]. To discriminate between *mef*(A) and *mef*(E), *mef* was amplified by PCR using primers MEFR (5′-CCAATGA TTTACACCGATT-3′), MEF1 (5′-AATACAACAATTGGAA ACTT-3′) and MEF2 (5′-AAGGAGTTGTGGTTCTGA-3′), as previously described (Gómez E, de la Pedrosa G, van der