Antimicrobial susceptibility patterns, *emm* type distribution and genetic diversity of *Streptococcus pyogenes* recovered in Brazil

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Streptococcus pyogenes *is responsible for a variety of infectious diseases and immunological complications. In this study, 91 isolates of* S. pyogenes *recovered from oropharynx secretions were submitted to antimicrobial susceptibility testing,* emm *typing and pulsed-field gel electrophoresis (PFGE) analysis. All isolates were susceptible to ceftriaxone, levofloxacin, penicillin G and vancomycin. Resistance to erythromycin and clindamycin was 15.4%, which is higher than previous reports from this area, while 20.9% of the isolates were not susceptible to tetracycline. The macrolide resistance phenotypes were* $cMLS_B$ *(10) and* $iMLS_B$ *(4) The* ermB *gene was predominant, followed by the* ermA *gene. Thirty-two* emm *types and subtypes were found, but five (*emm1, emm4, emm12, emm22, emm81*) were detected in 48% of the isolates. Three new* emm *subtypes were identified (*emm1.74, emm58.14, emm76.7*). There was a strong association between* emm *type and PFGE clustering. A variety of PFGE profiles as well as* emm *types were found among tetracycline and erythromycin-resistant isolates, demonstrating that antimicrobial resistant strains do not result from the expansion of one or a few clones. This study provides epidemiological data that contribute to the development of suitable strategies for the prevention and treatment of such infections in a poorly studied area.*

Key words: *Streptococcus pyogenes* - antimicrobial resistance - epidemiological typing - genetic diversity

Streptococcus pyogenes [Group A *Streptococcus* (GAS)] is one of the most clinically relevant species of *Streptococcus*. It has been associated with human infections ranging from mild sore throat and impetigo to invasive, life-threatening diseases, such as necrotising fasciitis and toxic shock syndrome. Moreover, immunological complications such as acute rheumatic fever, rheumatic heart disease and post-streptococcal acute glomerulonephritis are significant streptococcal disease burdens, especially in the developing world (Steer et al. 2009, Dale et al. 2013).

Several efforts have been made over the past decades to understand the epidemiology of GAS infections. Contributing to these efforts, molecular techniques such pulsed-field gel electrophoresis (PFGE) and multilocus sequencing typing have been extensively applied in the evaluation of the genetic relationships among isolates (Dicuonzo et al. 2001, Ardanuy et al. 2010). Another approach is *emm* typing, a sequence-based typing of the M protein (Beall et al. 1996). M protein is a cell surface protein that inhibits phagocytosis and is considered the most important epidemiological marker of GAS infections. This approach has the potential to type GAS isolates from different areas of the world. However, most available data are from high-income countries, which has lead to several limitations, such as the lack of knowledge of *emm* type prevalence in developing countries where severe streptococcal infections and immunological complications occur frequently (Steer et al. 2009).

The development of an efficient vaccine for GAS infections is another important issue. Several candidates have been evaluated, including the variable N-terminal and the conserved C-terminal epitopes of M protein (McNeil et al. 2005, Dale et al. 2011, Postol et al. 2013). Knowledge of the global distribution of *emm* types is required to formulate an N-terminal M protein-based vaccine that covers the prevalent M types in different geographical areas (Steer et al. 2009).

Treatment of GAS infections is based on beta-lactams because this species remains susceptible to penicillin G; macrolides, lincosamides and fluoroquinolones are recommended for allergic individuals. However, GAS resistance to these later antimicrobials has been described worldwide (Smeesters et al. 2009, Friães et al. 2012). While fluoroquinolone resistance in GAS is due to point mutations in the *gyr* and *par* genes, macrolide resistance is due to active efflux (M phenotype) or modification of the target site ($iMLS_B$ and $cMLS_B$ phenotypes) and is mediated by the genetic determinants *mefA/E*, *ermA* and *ermB* (D'Oliveira et al. 2003, Montes et al. 2010). It is worth noting that in some countries where macrolide resistance rates used to be high, such as Spain and China, unexpected reductions in the resistance rates have been recently observed (Huang et al. 2014, Montes et al. 2014).

Antimicrobial resistance rates and *emm* type distribution in a given population are essential to the development of suitable strategies for the prevention and treatment of GAS infections. The aim of this study was to investigate the antimicrobial susceptibility and *emm* type and to evaluate the genetic diversity of circulating GAS isolates in the metropolitan area of Rio de Janeiro, Brazil.

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SUBJECTS, MATERIALS AND METHODS

Isolates - Ninety-one GAS isolates recovered from oropharynx secretions were included in this study. Clinical specimens were processed during routine diagnoses by one clinical laboratory in Rio de Janeiro from January 2008-July 2012. The subjects' ages varied from two-56 years, but 46% of the isolates were recovered from children two-11 years of age and 51.6% were from women. In our laboratory, these isolates were cultured on blood agar plates (Difco Laboratories, USA) and submitted to conventional tests (PYR test, bacitracin susceptibility and streptococcal serogrouping) to confirm species identification.

Antimicrobial susceptibility testing - All isolates were submitted to susceptibility tests to ceftriaxone (30 µg), clindamycin (2 µg), erythromycin (15 µg), levofloxacin (5 µg), penicillin (10 U), tetracycline (30 µg) and vancomycin (30 µg) (CECON, Brazil) using the disk diffusion method on Mueller-Hinton blood agar (Difco) according to CLSI guidelines (2013). Macrolide resistance phenotypes were determined by the double disk test using erythromycin (15 µg) and clindamycin (2 µg) disks placed 12 mm apart (CLSI 2013). The erythromycin minimum inhibitory concentration (MIC) was determined in all resistant and intermediate isolates by the agar dilution method (CLSI 2009).

Investigation of erythromycin resistance-associated genes - DNA preparation was performed as previously described (Dmitriev et al. 2002) with modifications. Briefly, suspensions with turbidity adjusted to McFarland Standard 3.0 were prepared in 300 µL of 10 mM Tris-EDTA buffer and boiled for 5 min. The presence of *ermA*, *ermB* and *mefA/E* genes was investigated in erythromycin-resistant isolates using specific polymerase chain reaction (PCR) protocols (Sutcliffe et al. 1996, Perez-Trallero et al. 2007). Cycling was carried in a GeneAmp 9700 Thermocycler (Applied Biosystems, USA). PCR products were resolved on 1% agarose gels.

Determination of emm types - *emm* types were determined by a sequence-based protocol (cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm) using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed using a 3130 Genetic Analyzer (Applied Biosystems). Sequences were edited using Bioedit software v.7.0 and compared with reference sequences using the BLAST algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences that did not match with 100% similarity to any sequence deposited in GenBank were submitted to the Centers for Disease Control and Disease (CDC) *emm* sequence database (cdc.gov/ncidod/biotech/strep/strepblast.htm) for assignment to new *emm* subtypes.

Analysis of DNA restriction patterns by PFGE - All 44 isolates belonging to the five prevalent *emm* types (*emm1*, *emm4*, *emm12*, *emm22* and *emm81*) were analysed by PFGE after the DNA was digested with *SmaI* according to a previous protocol (Teixeira et al. 1997) with modifications as described below. Briefly, bacteria were grown on blood agar plates. A 300-µL aliquot of bacterial suspension in PIV buffer was mixed with an equal volume of low melting point agarose (Promega, USA) and distributed into plug moulds. Plugs were incubated in 2 mL of lysis solution containing 5 mg/mL lysozyme. *Sma*I digestion was performed according to the manufacturer's recommendations (Invitrogen, USA). DNA fragments were separated by the CHEF-DRIII system (Bio-Rad Laboratories, USA). The dice coefficient was calculated by visual analysis and dendrograms based on Unweighted Pair Group Method with Arithmetic Mean were constructed using genomes.urv.cat/UPGMA.

Statistical analyses - The discriminatory power of *emm* typing, regarding the overall population as well as erythromycin and tetracycline resistance, was measured using Simpson's index of diversity (SID) by calculating the 95% confidence intervals (CI) (Hunter & Gaston 1988, Grundmann et al. 2001). All calculations were performed using the Comparing Partitions Tool available from comparingpartitions.info.

RESULTS

Conventional tests identified all isolates as S. pyogenes. By the disk diffusion method, all isolates were susceptible to ceftriaxone, levofloxacin, penicillin G and vancomycin. Tetracycline-resistant and intermediate isolates comprised 18.7% and 2.2% of isolates, respectively. Clindamycin resistance was observed in 15.4% of isolates, while erythromycin-resistant and intermediate isolates comprised 14.3% and 1.1% of isolates, respectively. The erythromycin MIC varied from 8-256 µg/mL and therefore, the resistance rate was 15.4%. Ten and four isolates showed $cMLS_B$ and $iMLS_B$ resistance phenotypes, respectively. The genetic determinant *ermB* was predominant and was detected in 78.6% of the erythromycin-resistant isolates, alone or in association with *ermA* (64.3%). Neither the M phenotype nor the *mefA/E* gene was observed in this study. Erythromycin MIC values, the distribution of macrolide resistance phenotypes and genotypes and *emm* types of erythromycin-resistant isolates are shown in Table.

Thirty-two *emm* types or subtypes were identified among 86 of the 91 GAS isolates. Five isolates were non-typeable, even after three attempts. The most frequent

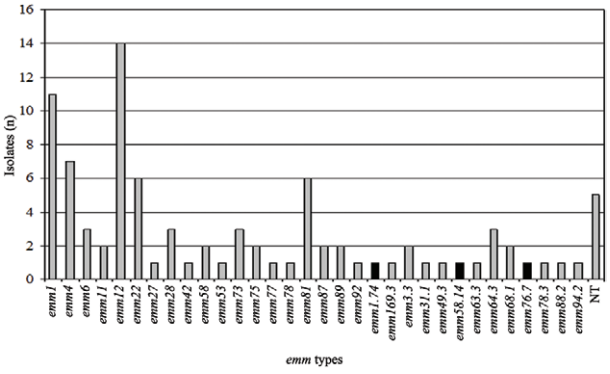

Fig. 1: distribution of *emm* types and subtypes among isolates of *Streptococcus pyogenes* recovered from oropharynx secretion. NT: non-typeable; ■: new subtypes.

TABLE

Phenotypic and genotypic characteristics of macrolide resistant isolates

Isolate number	Year of recovery	*emm* type	MIC (μg/mL)	Phenotype	Macrolide resistance genes		
					ermA	*ermB*	*mefA/E*
186	2008	68.1	> 256	$cMLS_B$	-	+	-
274	2008	58	> 256	$iMLS_B$	+	-	-
302	2008	58	> 256	$iMLS_B$	+	-	-
402	2009	1	> 256	$cMLS_B$	-	+	-
418	2009	58.14	8	$iMLS_B$	+	-	-
425[a]	2009	3.3	16	$cMLS_B$	-	+	-
536	2010	22	> 256	$cMLS_B$	-	+	-
637A	2011	1	> 256	$cMLS_B$	+	+	-
638	2011	76.7	> 256	$cMLS_B$	+	+	-
710	2011	28	> 256	$cMLS_B$	-	+	-
749	2011	73	> 256	$cMLS_B$	+	+	-
750	2011	6	> 256	$cMLS_B$	+	+	-
780	2012	11	> 256	$cMLS_B$	+	+	-
798	2012	11	> 256	$iMLS_B$	+	+	-

a: this isolate was intermediate by disk diffusion; $cMLS_B$: constitutive MLS_B phenotype; $iMLS_B$: inducible MLS_B phenotype; MIC: minimal inhibitory concentration (susceptible < 0.25 μg/mL, intermediate 0.5 μg/mL, resistant > 1 μg/mL); +: presence of the gene; -: absence of the gene.

types were *emm1*, *emm4*, *emm12*, *emm22* and *emm81*, accounting for 48% of all isolates. Three new sequences were designated by the CDC *Streptococcus* Laboratory as new *emm* subtypes (*emm1.74*, *emm58.14* and *emm76.7)* and deposited in the *emm* sequence database (cdc.gov/pub/infectious_diseases/biotech/tsemm) and in GenBank (accession KM364527-KM364529). These new subtypes differed from their parental types in 1-2% of the nucleotide sequence. The *emm* typing revealed a high level of diversity among the overall population (SID = 0.941; 95% CI, 0.917-0.965). The frequency of each *emm* type is shown in Fig. 1.

Fourteen different *emm* types or subtypes were found among 19 tetracycline non-susceptible isolates, while 11 distinct *emm* types or subtypes were found among 14 erythromycin and clindamycin-resistant isolates. Two new *emm* subtype isolates were resistant to these antimicrobials. The SID values calculated for erythromycin-resistant (0.967; 95% CI, 0.929-1.000) and tetracycline non-susceptible (0.959; 95% CI, 0.915-1.000) isolates were higher than those calculated for erythromycin (0.929; 95% CI, 0.898-0.960) and tetracycline (0.898; 95% CI, 0.855-0.940) susceptible isolates.

PFGE analysis of 44 isolates belonging to the five prevalent *emm* types generated 20 restriction profiles, which are shown in Fig. 2. One single PFGE profile was shared by all *emm12* isolates. Three profiles, whose similarities varied from 50-85%, were observed among *emm1* isolates. The most frequent profile was shared by eight isolates, including one resistant to erythromycin. Regarding *emm4*, four profiles were observed among seven isolates, ranging from 85-96% similarity. Among *emm22* and *emm81* isolates (6 isolates each), unique PFGE profiles were observed for each isolate, varying from 70-95% similarity.

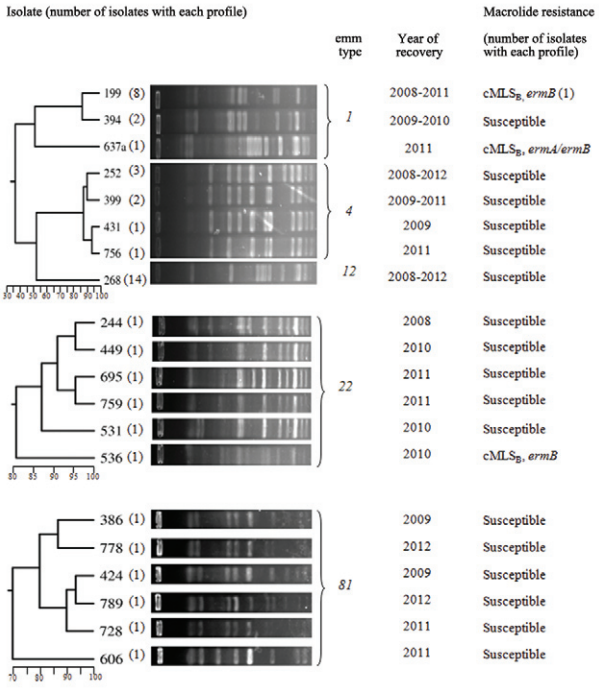

Fig. 2: pulsed-field gel electrophoresis profiles, dendrograms, year of isolation and macrolide resistance features of prevalent *emm* types isolates.

DISCUSSION

In this study, 91 GAS isolates recovered from oropharynx secretions of residents of Rio de Janeiro were submitted to susceptibility testing and typing methodologies. Despite the lack of data regarding the clinical conditions of the individuals, these results are relevant because both infected and colonised individuals can transmit this species to susceptible subjects.

Isolates were fully susceptible to beta-lactams and glycopeptides, as observed over decades of antibiotic usage. They were also susceptible to levofloxacin, despite previously detected resistance to this antimicrobial (Smeesters et al. 2009, Montes et al. 2010).

The tetracycline resistance rate was lower than that observed in a study conducted in Brazil one decade ago, in which the authors reported 43.1% resistance (D'Oliveira et al. 2003). Instead, it was very similar to recent data from southern Brazil and Portugal (Torres et al. 2011, Friães et al. 2012). However, 73% resistance to this antimicrobial was reported in a recent study from India (Mathur et al. 2014). These findings may reflect that, while a consistent trend of decreasing tetracycline resistance has been recently observed in Brazil, circulating GAS isolates from other regions are highly resistant to this antimicrobial.

Regarding erythromycin and clindamycin resistance, the rates found here far exceeded those previously reported in the same geographical area (D'Oliveira et al. 2003, Torres et al. 2011), but they are very similar to those described in some recent studies conducted in Europe (Friães et al. 2012, Syrogiannopoulos et al. 2013). While a trend of decreasing erythromycin resistance has been recently observed in Spain and Taiwan (Huang et al. 2014, Montes et al. 2014), the resistance rates in our study fluctuated over the years. Inducible and constitutive MLS_B phenotypes, which are associated with *ermA* and/or *ermB* genes, were observed among isolates belonging to a variety of *emm* types, reflecting the polyclonal origin of such isolates. It is worth noting that the M phenotype and the *mefA/E* genotype, associated with *emm12*, were common in Brazil before the year 2000 (Torres et al. 2011), but they have not been detected since that time, either by those authors or in this study. In contrast, MLS_B emerged after the 2000s and is linked to a variety of *emm* types, such as *emm11*, *emm22*, *emm28* and *emm73* (Torres et al. 2011). In contrast to this local replacement of the M phenotype with the $cMLS_B$ phenotype, the M phenotype has been prevalent over 12 years of survey (1998-2010) in Taiwan (Huang et al. 2014). However, the authors observed the replacement of *emm12* with *emm22* as the prevalent type associated with macrolide resistance, as well as a decreasing rate in macrolide resistance from 53.1% before 2000 to 10.7% from 2006-2010. These variations demonstrate how dynamic the bacterial population is worldwide and also highlight the need for changing therapeutic recommendations in geographical areas where MLS_B phenotypes predominate.

A wide variety of *emm* types, including new alleles, were observed in our study, as demonstrated by the SID calculation. The diversity of *emm* types and the detection of new alleles from GAS isolates recovered from oropharynx secretions have been described in previous studies from Brazil (Teixeira et al. 2001, Tartof et al. 2010) and throughout the world (Shea et al. 2011). These findings reflect the genetic heterogeneity of such isolates and contribute to the expansion of the *emm* type database. The most frequent *emm* types found here are among the prevalent types in high-income countries, where the majority of studies have been performed (Steer et al. 2009, Shea et al. 2011). However, they differ significantly from those types observed in recent studies from India and the Pacific Region (Baroux et al. 2014, Mathur et al. 2014). Due to the limited amount of data from Latin America, our results contribute to a better understanding of *emm* type distribution in a poorly studied area. This issue is particularly important considering that M protein-based vaccines are under development (Dale et al. 2013). When comparing the two most promising 26 and 30-valent M vaccines, 49% and 64% of our isolates, respectively, have *emm* types included in these formulations. The potentially low impact of these vaccines can be explained by the fact that the M protein fragments for the 26-valent M vaccine were selected based on *emm* type distribution in North America (Steer et al. 2009).

There was strong agreement between *emm* type and PFGE clustering, as previously observed (Torres et al. 2011). No identical PFGE profile was observed among isolates belonging to distinct *emm* types, which could be related to the horizontal transfer of *emm* genes (Whatmore et al. 1994). Regarding erythromycin-resistant isolates, a great diversity of PFGE profiles and *emm* types were observed, illustrating that this characteristic is not due to the expansion of a specific clone. Moreover, both erythromycin-susceptible and erythromycin-resistant *emm1* isolates shared a single PFGE profile. This finding suggests that the *emm* type, not erythromycin resistance, is more likely to be a determinant of clonality.

In conclusion, susceptibility testing and epidemiological typing techniques revealed an incidence of macrolide resistance not yet observed in this area and a great diversity of *emm* types, including new alleles, among GAS isolates circulating in Brazil. These data contribute to the improvement of prevention and treatment strategies of GAS infections.

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