Original Article

Polyclonal dissemination of tetracycline resistance among *Streptococcus pyogenes* paediatric isolates from Brazil

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Abstract

Introduction: Scarce data are available on Group A *Streptococcus* (GAS) antibiotic resistance in South America.
Methodology: The antibiotic susceptibility patterns of GAS recovered from symptomatic children living in the central part of Brazil during a prospective epidemiological study were analyzed.
Results: No isolates were resistant to penicillin or macrolides. Sixty-one percent of the isolates were highly resistant to tetracycline, of which 85% harboured the *tetM* resistance gene. Ninety-five percent of these tetracycline resistant isolates were also resistant to minocycline. Thirty different *emm*-types were associated with tetracycline resistance. Phylogenetic analysis indicates that tetracycline resistance arose independently in distantly related *emm*-types.
Conclusions: A high level of GAS tetracycline resistance has been observed in the central part of Brazil due to the polyclonal dissemination of resistant *emm*-types.

Key words: tetracycline resistance; minocycline resistance; *emm* gene; GAS phylogeny

J Infect Dev Ctries 2010; 4(11):704-711.

(Received 21 April 2010- Accepted 7 July 2010)

Introduction

Streptococcus pyogenes, also known as Group A *Streptococcus* (GAS), is one of the most common paediatric pathogens. It is responsible for a wide spectrum of human diseases, ranging from localized (pharyngitis, impetigo) to lethal infections (toxic shock syndrome, necrotizing fasciitis) [1,2]. Penicillin represents the drug of choice for treating GAS infections, with erythromycin being used in cases of beta-lactam allergy [3]. GAS resistance to macrolides is increasing in many countries, ranging from 6.2% in North America to more than 20% in some European countries [4]. However, great variability across different areas has been reported [5] and scarce data are available for GAS isolated in South America [6-9]. GAS tetracycline resistance is also reported in many countries and data from South America show a proportion of resistant isolates ranging from 8 to 70% [10-13]. None of these recent studies have analyzed the presence of the tetracycline resistance-conferring genes.

In Brazil, antibiotics can be purchased without any prescription in most of the pharmacies and self-medication is common, even in children [14]. A study performed in Santa Catarina (South Brazil) in 2002 showed that 74% of the pharmacies offered antibiotic without any prescription [15]. This is a cause for concern as an increased consumption of antibiotics, such as erythromycin and tetracycline, correlates with an increase of resistance among GAS [16,17].

Two main well-described molecular mechanisms are responsible for macrolide resistance in GAS. The *ermA*, *ermB* and *ermTR* resistance genes encode enzymes that methylate the 23S ribosomal RNA [18]. This target site modification confers cross-resistance to lincosamide, streptogramines B, and other macrolides [18]. The other molecular mechanism is encoded by the *mefA* gene which constitutes an active

drug efflux pump and confers resistance to 14- and 15- membered macrolides. A similar mechanism is encoded by the *tetK* and *tetL* genes which confer tetracycline resistance to GAS. Tetracycline and minocycline GAS co-resistance, on the other hand, relies mostly on the *tetM* gene, and to a lesser extent on the *tetO* gene, both encoding ribosomal protection proteins [19].

emm-typing is the GAS molecular typing gold standard and is based on the DNA sequence of the 5' end 180 nucleotides of the *emm* gene [1]. Currently, more than 200 *emm*-types have been reported throughout the world [20]. This method of classification correlates quite well with the expression of a streptococcal apoproteinase called the serum opacity factor (*sof*) [21].

We previously published the clinical and microbiological characterization (*emm*-typing) of a GAS paediatric collection recovered from symptomatic children in the central part of Brazil during the year 2004 [22]. The aim of the present study was to characterize the penicillin, macrolide, and tetracycline susceptibility patterns in this collection, as well as the genetic determinants underlying said resistance.

Methodology

Materials

GAS clinical isolates analyzed in this study were prospectively collected in 2004 in Brasília, Brazil, from symptomatic children [22]. Patients of <1 to 15 years old attending three public hospitals of Brasília were included in this study. The ethical board of all participant hospitals approved this study. Isolates were mostly recovered from pharyngitis (44%) and cutaneous infections (48%). Beta-haemolytic streptococci were phenotypically identified by beta-haemolysis on blood agar, colony morphology, Gram stain, catalase reaction, and susceptibility to 0.04 U bacitracin. GAS identification was performed with a positive latex agglutination test containing group A specific antisera (Slidex, BioMérieux, Paris, France). *emm*-typing was performed using the Centre for Disease Control (CDC) protocol as previously described in [22] (information are available at http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). The phylogenetic analysis of M protein was published previously [23,24]. Briefly, the portion of the *emm* gene encoding the whole surface exposed portion of M protein (including hypervariable and conserved regions) was sequenced, translated into an amino acid sequence, and a multiple alignment was performed using Clustal W [25]. The sequence alignments were loaded in *MEGA* version 4 [26] to generate a bootstrapped tree using the neighbour-joining algorithm [27].

sof *genotype*

Isolates within a given *emm*-type usually display the same opacity factor genotype [28-31]. The opacity factor genotype was deduced from epidemiological data collected in several studies [29-31].

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) for penicillin, erythromycin, azithromycin, clindamycin, telithromycin, tetracycline, and minocycline were determined by a reference broth microdilution method (Merlin-Diagnostika, Bornheim-Hersel, Germany) in cation-adjusted Mueller–Hinton broth (Difco Laboratories, Detroit, MI, USA) supplemented with 4.6% lysed horse blood as recommended by the Clinical Laboratory Standards Institute (CLSI) [32]. *Streptococcus pneumoniae* ATCC 49619 was used as a control. Breakpoints for penicillin, erythromycin, azithromycin, clindamycin, and tetracycline were those described by the CLSI (Table 1). Breakpoints for telithromycin were $\leq$ 1 μg/mL for susceptibility and $\geq$ 4 μg/mL for resistance and for minocycline were $\leq$ 2 μg/mL for susceptible and $\geq$ 8 μg/mL for resistant.

Detection of resistance genes by PCR

Four tetracycline resistance genes (*tetL*, *tetK*, *tetM* and *tetO*) were detected by polymerase chain reaction (PCR) as previously described [33]. *Streptococcus pyogenes* BM137, *Enterococcus faecalis* BM4110, *Staphylococcus aureus* R16794, and *Enterococcus faecalis* P33 were used as positive controls for *tetM*, *tetO*, *tetK*, and *tetL* detection, respectively.

Results and Discussion

One hundred and thirty GAS isolates were recovered in Brasilia, Brazil, in 2004 from both pharyngitis and skin infections in equal proportion [22]. None of these isolates were resistant to penicillin. The MICs for penicillin were very low, ranging from 0.004 mg/L to 0.032 mg/L for 98.5% of the isolates (Table 1). All the isolates were susceptible to the tested macrolides, lincosamides, and ketolides (Table 1). On the contrary, 79 of 130 isolates (61%) were resistant to tetracycline, most of

them (75/79; 95%) presenting a high level of resistance (MIC ≥ 32mg/L). No difference in antibiotic resistance patterns could be observed among the three participating centres. This high rate of tetracycline resistance and absence of macrolide resistance is similar to the results obtained in previous studies in other areas of South America, including Brazil [10-12] (Table 2). This may be related to the low prescription rate of macrolides in Brazilian public hospitals; less than 3% of the acute pharyngitis were treated with macrolides in our study [34]. Ninety-five percent (75/79) tetracycline resistant strains were also resistant to minocycline (MIC ≥ 8 mg/L) and all the minocycline resistant isolates (*n* = 75) were also tetracycline resistant. The remaining four isolates resistant to tetracycline (MIC = 16 mg/L) showed an intermediate level of minocycline resistance (MIC = 2 mg/L). Forty-seven out of the 79 tetracycline-resistant isolates (60%) were skin isolates; the remaining isolates were from the throat.

The *tetM* tetracycline resistance gene was found in 87% of the resistant isolates (69/79). None of the resistant isolates were positive for any of the three other tetracycline resistance genes (*tetL*, *tetK* and *tetO*) tested in this study. The *tetM* positive GAS isolates displayed tetracycline MICs ranging from 32 to ≥256 mg/L and minocycline MICs ranging from 8 to 16 mg/L (Table 3). In the other 13% (10/79 isolates), none of the four resistance genes were detected, suggesting that others or yet undescribed genes might be involved in tetracycline and minocyclin resistance in these isolates. These isolates belonged to four different *emm*-types (*emm* 33, 44/61, 64, and 74). Tetracycline and minocycline MICs ranged from 16 to 32 mg/L and from 2 to 16 mg/L, respectively (Table 3). Thus, among the 10 tetracycline-resistant isolates negative for *tetM*, four were minocycline intermediate, which indicates that the tetracycline resistance mechanism might be via an efflux pump, while the remaining six were minocycline resistant, suggesting the presence of a ribosomal methylase encoding gene [19].

To gain insights on the tetracycline resistance distribution with respect to the genetic relationships shared by these GAS isolates, we took advantage of our previous studies describing the genetic relatedness of the extracellular portion of the M protein among our isolates [23,35]. Figure 1 shows that the GAS isolates included in this study separated into two main clades reflecting their *emm*-pattern and *sof* status [28,29,36,37]. The *sof*$^+$ A clade was mainly composed of *emm*-pattern E isolates, which are considered to be generalist GAS (skin and throat tropism) [36]. This clade contained the majority of the isolates (75/130, 58%). Clade B, on the other hand, contained 51/130 isolates (39%) and was composed of *sof*$^+$ isolates belonging to *emm*-pattern A-C (throat) and D (skin) [36]. Four isolates were not included in the phylogenetic analysis for technical reasons [23]. The 51 tetracycline susceptible isolates belonged to 21 different *emm*-types, which were found both in clade A and B. Interestingly, the 79 tetracycline resistant isolates were also found in these two clades. They represented 30 different *emm*-types and were of the A-C, D and E *emm*-pattern groups. This observation illustrates that tetracycline resistance does not show any preferential genetic background and may arise in distantly related *emm*-types. The Tn*916* transposon is widely distributed in Gram-positive bacteria [38] and carries the *tetM* gene [39]. Multiclonal dissemination of the resistance trait is likely to be caused by horizontal transfer of the transposon (like the Tn*916*) rather than the epidemic spread of a few resistant clones. Multiclonal dissemination of tetracycline resistance was also described in southeast Brazil [10,11] and in Iran [17], which is in accordance with our results. However, other studies have observed somewhat different results. In Italy, 80% of the tetracycline resistant GAS isolates were found in four closely related clusters, as defined by pulsed field gel electrophoresis (PFGE) [40]. However, GAS epidemiological studies underlined the small number of circulating *emm*-types in western countries as opposed to "developing/emerging countries" where a large number of different *emm*-types are usually recovered [41]. The apparent clonality of tetracycline resistance observed in western countries might in fact be due to the reduced number of circulating *emm*-types rather than the non-susceptible status. A similar interpretation has been proposed for fluoroquinolone non-susceptibility in GAS [24].

Although extensive genetic diversity is found in tetracycline resistant GAS, the same *emm*-type is rarely associated with susceptible and resistant isolates. The closely related *emm*58 and *emm*118 *emm*-types (clade A) have representatives that are either susceptible or resistant to tetracycline. The unrelated *emm*74 (clade B) and *emm*44/61 (clade A) have representatives that are tetracycline and minocycline resistant or tetracycline resistant and minocycline susceptible. The remaining 42 *emm*-types have representatives that are exclusively

tetracycline resistant or tetracycline susceptible. Thus we observed a good correlation between defined *emm*-types and tetracycline susceptibility status. At least three previous studies (performed in Italy, Iran, and Brazil respectively) observed a somewhat different correlation. These authors found that susceptible and resistant isolates belong to the same clonal type, as determined by PFGE [10,17,40]. Within a defined geographical location and a restricted period of time (*i.e.* the settings of this study), *emm*-typing usually correlates quite well with other typing methods, such as multilocus sequence typing or PFGE [28,42]. In contrast, when comparing different locations or longer periods of time (*i.e.* the settings of the three other studies), identical *emm*-types might be associated with different clonal types. This difference in the study design might therefore explain the different observations. Studies using different typing methods might help to clarify the correlation between *emm*-types, clonal types, and tetracycline/minocycline susceptibility status.

Table 1. *In vitro* activities of 7 antibiotics

Antibiotic	No. of isolates with MIC (mg/L)																	No. (%) of resistance
	0.004	0.008	0.016	0.032	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
Penicillin	124	2	1	1	0	2	0	0	/	/	/	/	/	/	/	/	/	0 (0)
Erythromycin	/	/	/	1	48	80	1	0	0	0	0	0	0	0	0	0	/	0 (0)
Azithromycin	/	/	/	63	25	39	3	0	0	0	0	0	0	0	0	0	/	0 (0)
Clindamycin	/	/	/	7	98	25	0	0	0	0	0	0	0	0	0	0	/	0 (0)
Telithromycin	/	/	/	129	1	0	0	0	0	0	0	0	0	0	/	/	/	0 (0)
Tetracycline	/	/	/	/	0	7	43	1	0	0	0	0	4	49	24	1	1	79 (61)
Minocycline	/	/	/	/	1	36	14	0	0	4	0	47	28	0	0	/	/	75 (58)

Susceptibility/Resistance breakpoints: Penicillin ≤0.12/NA, Erythromycin ≤0.25/≥1, Azithromycin ≤0.5/≥2, Clindamycin ≤0.25/≥1, Telithromycin ≤1/≥4, Tetracycline ≤0.25/≥8, Minocycline ≤0.25/≥8

Table 2. Macrolide and tetracycline resistance among GAS isolated in South America

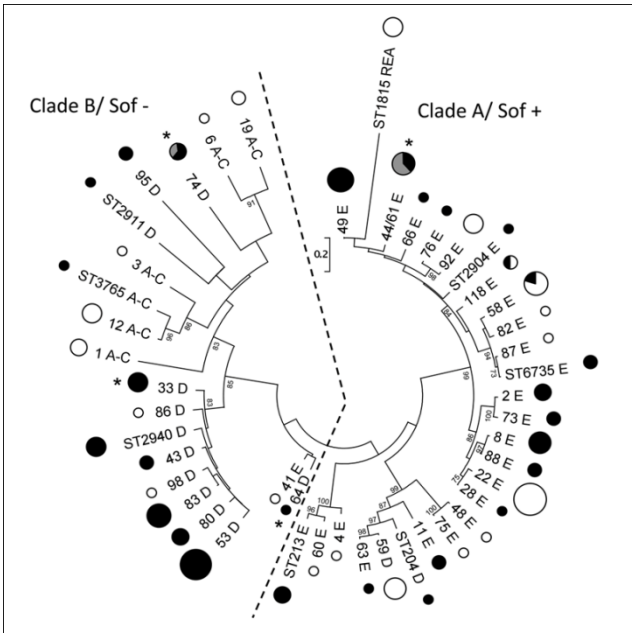
Year	**Location**	**No. of isolates**	**% of erythromycin resistance**	**Presence of erythromycin resistance genes**	**% of tetracycline resistance**	**Genetic diversity among resistant isolates**	**Ref**
1990-1998	Santiago, Chile	594	7.2	ND	ND	'High' (15 PFGE pattern in 28 ery R isolates)	[43]
1978-1997	Southeast, Brazil	211	0	ND	50	'High' (60 PFGE pattern in 90 tet R isolates)	[10]
1995-1999	Southeast, Brazil	37	0	ND	70	'High' (11 *emm*-types in 26 tet R isolates)	[11]
1994-1999	Rio de Janeiro, Brazil	357	1	*mefA* and *ermTR*	43	ND	[12]
1999-2000	Buenos Aires, Argentina South and center, Brazil Mexico, Mexico	33 145 99	12.1 5.5 11.1	ND	21.2 24.8 8.1	ND	[13]
1999-2001	Buenos Aires, Argentina	1626	6.7	ND	ND	ND	[44]
1999-2000	Buenos Aires, Argentina	568	10	*mefA* (96%)	ND	ND	[45]
2002-2003	Bariloche, Argentina	1068	2.4	*mefA* (84.6%)	ND	ND	[46]

Studies including at least 20 GAS isolates recovered in South America and published after January 2000 were compiled. ND: not determined. None of these studies analysed the presence of tetracycline resistance genes. PFGE, Pulsed field gel electrophoresis; ery R, erythromycin resistant; tet R, tetracycline resistant.

Table 3. Presence of *tetM* according to tetracycline and minocycline MICs and *emm*-type

Tetracycline MIC	Minocycline MIC	*emm*-type	No. of isolates	*tetM*
16	2	44/61	3	-
		74	1	-
32	8	11	2	+
		2	3	+
		28	1	+
		33	3	-
		43	1	+
		44/61	2	+ and -
		49	3	+
		60	1	+
		64	1	-
		73	1	+
		74	2	+
		80	1	+
		83	2	+
		88	2	+
		93	1	+
		st213	1	+
		st2911	1	+
		st2940	1	+
		st3765	1	+
	16	33	1	-
		49	4	+
		53	1	+
		58	1	+
		63	1	+
		73	1	+
		8	3	+
		80	1	+
		83	1	+
		95	1	+
		st204	1	+
		st213	2	+
		st2940	1	+
64	8	43	1	+
		53	1	+
		83	2	+
		st2904	1	+
		st2940	1	+
	16	118	1	+
		53	8	+
		76	1	+
		8	2	+
		80	1	+
		83	1	+
		st2940	1	+
		st6735	2	+
		nt	1	+
128	16	53	1	+
≥ 256	8	95	1	+

Figure 1. Tetracycline non-susceptibility arise in distantly related *emm*-types



The genetic relationships between the surface-exposed region of the M proteins of 46 *emm*-types included in this study are represented. The *emm*-types and *emm*-patterns are indicated. The surface of the circle is proportional to the relative frequency of each *emm*-type. The colour of the circle indicates the tetracycline resistance status: white stands for tetracycline and minocycline susceptible, grey for tetracycline resistant and minocycline susceptible and black for tetracycline and minocycline resistant isolates. The stars indicate the *emm*-types in which none of the 4 tetracycline resistance genes was detected. The evolutionary history was inferred using the neighbour-joining method [27]. The tree is drawn to scale, with branch lengths proportional to the evolutionary distances. Bootstrap value higher than 70% are shown next to the branches (500 replicates).

Conclusions

Our epidemiological study shows that the GAS isolates included in our study are macrolide susceptible. However, a high frequency of tetracycline resistance was observed (61 %) and is likely to be caused by a polyclonal dissemination of the *tetM* resistance gene. Tetracycline resistance has been suggested to precede macrolide resistance [47]. With the potential increase of macrolide prescriptions in the future, active epidemiological surveillance will be needed to monitor the evolution of GAS antibiotic resistance patterns.

Acknowledgments

This study was performed with the support of an academic exchange program between the Université Libre de Bruxelles (Belgium) and the Universidade de Brasília (Brazil). We thank all the professionals of the three participant hospitals for their kind collaboration. We thank Horacio Lopardo and Gabriel Gutkind for kindly sharing their data on GAS antibiotic resistance in Argentina. We thank Surbhi Malhotra-Kumar and Herman Goossens for providing the control strains for PCR. PRS is 'chargé de recherche' FRNS and has been supported by an ESPID fellowship award.

References

1. Bisno AL, Brito MO, Collins CM (2003) Molecular basis of group A streptococcal virulence. Lancet Infect Dis 3: 191-200.
2. Smeesters PR, Dreze PA, Perez-Morga D, Biarent D, Van Melderen L, Vergison A (2010) Group A Streptococcus virulence and host factors in two toddlers with rheumatic fever following toxic shock syndrome. Int J Infect Dis 14: e403-409.
3. Bisno AL, Gerber MA, Gwaltney JM, Jr., Kaplan EL, Schwartz RH (2002) Practice guidelines for the diagnosis and management of group A streptococcal pharyngitis. Infectious Diseases Society of America. Clin Infect Dis 35: 113-125.
4. Montagnani F, Stolzuoli L, Croci L, Rizzuti C, Arena F, Zanchi A, Cellesi C (2009) Erythromycin resistance in Streptococcus pyogenes and macrolide consumption in a central Italian region. Infection 37: 353-357.
5. Freeman AF, Shulman ST (2002) Macrolide resistance in group A Streptococcus. Pediatr Infect Dis J 21: 1158-1160.
6. Robinson DA, Sutcliffe JA, Tewodros W, Manoharan A, Bessen DE (2006) Evolution and global dissemination of macrolide-resistant group A streptococci. Antimicrob Agents Chemother 50: 2903-2911.
7. Richter SS, Heilmann KP, Beekmann SE, Miller NJ, Miller AL, Rice CL, Doern CD, Reid SD, Doern GV (2005) Macrolide-resistant Streptococcus pyogenes in the United States, 2002-2003. Clin Infect Dis 41: 599-608.
8. Grivea IN, Al-Lahham A, Katopodis GD, Syrogiannopoulos GA, Reinert RR (2006) Resistance to erythromycin and telithromycin in Streptococcus pyogenes isolates obtained between 1999 and 2002 from Greek children with tonsillopharyngitis: phenotypic and genotypic analysis. Antimicrob Agents Chemother 50: 256-261.
9. Strakova L, Motlova J, Jakubu V, Urbaskova P, Kriz P (2007) Emergence of a novel macrolide-resistant Streptococcus pyogenes emm53 strain. Clin Microbiol Infect 13: 443-445.
10. de Melo MC, Sa Figueiredo AM, Ferreira-Carvalho BT (2003) Antimicrobial susceptibility patterns and genomic diversity in strains of Streptococcus pyogenes isolated in 1978-1997 in different Brazilian cities. J Med Microbiol 52: 251-258.
11. Teixeira LM, Barros RR, Castro AC, Peralta JM, Da Gloria SCM, Talkington DF, Vivoni AM, Facklam RR, Beall B (2001) Genetic and phenotypic features of Streptococcus pyogenes strains isolated in Brazil that harbor new emm sequences. J Clin Microbiol 39: 3290-3295.
12. d'Oliveira RE, Barros RR, Mendonca CR, Teixeira LM, Castro AC (2003) Antimicrobial susceptibility and survey of macrolide resistance mechanisms among Streptococcus pyogenes isolated in Rio de Janeiro, Brazil. Microb Drug Resist 9: 87-91.
13. Mendes C, Marin ME, Quinones F, Sifuentes-Osornio J, Siller CC, Castanheira M, Zoccoli CM, Lopez H, Sucari A, Rossi F, Angulo GB, Segura AJ, Starling C, Mimica I, Felmingham D (2003) Antibacterial resistance of community-acquired respiratory tract pathogens recovered from patients in Latin America: results from the PROTEKT surveillance study (1999-2000). Braz J Infect Dis 7: 44-61.
14. Pereira FS, Bucaretchi F, Stephan C, Cordeiro R (2007) Self-medication in children and adolescents. J Pediatr (Rio J) 83: 453-458.

15. Volpato DE, de Souza BV, Dalla Rosa LG, Melo LH, Daudt CA, Deboni L (2005) Use of antibiotics without medical prescription. Braz J Infect Dis 9: 288-291.
16. Bergman M, Huikko S, Pihlajamaki M, Laippala P, Palva E, Huovinen P, Seppala H (2004) Effect of macrolide consumption on erythromycin resistance in Streptococcus pyogenes in Finland in 1997-2001. Clin Infect Dis 38: 1251-1256.
17. Jasir A, Tanna A, Noorani A, Mirsalehian A, Efstratiou A, Schalen C (2000) High rate of tetracycline resistance in Streptococcus pyogenes in Iran: an epidemiological study. J Clin Microbiol 38: 2103-2107.
18. Malhotra-Kumar S, Lammens C, Chapelle S, Wijdooghe M, Piessens J, Van Herck K, Goossens H (2005) Macrolide- and telithromycin-resistant Streptococcus pyogenes, Belgium, 1999-2003. Emerg Infect Dis 11: 939-942.
19. Shlaes DM (2006) An update on tetracyclines. Curr Opin Investig Drugs 7: 167-171.
20. Smeesters PR, McMillan DJ, Sriprakash KS (2010) The streptococcal M protein: a highly versatile molecule. Trends Microbiol 18: 275-282.
21. Cunningham MW (2000) Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 13: 470-511.
22. Smeesters PR, Vergison A, Campos D, de Aguiar E, Miendje Deyi VY, Van Melderen L (2006) Differences between Belgian and Brazilian group A Streptococcus epidemiologic landscape. PLoS ONE 1: e10.
23. Smeesters PR, Mardulyn P, Vergison A, Leplae R, Van Melderen L (2008) Genetic diversity of Group A Streptococcus M protein: implications for typing and vaccine development. Vaccine 26: 5835-5842.
24. Smeesters PR, Vergison A, Junior DC, Van Melderen L (2009) Emerging fluoroquinolone-non-susceptible group A streptococci in two different paediatric populations. Int J Antimicrob Agents 34: 44-49.
25. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.
26. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol 24: 1596-1599.
27. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.
28. McGregor KF, Spratt BG, Kalia A, Bennett A, Bilek N, Beall B, Bessen DE (2004) Multilocus sequence typing of Streptococcus pyogenes representing most known emm types and distinctions among subpopulation genetic structures. J Bacteriol 186: 4285-4294.
29. Beall B, Gherardi G, Lovgren M, Facklam RR, Forwick BA, Tyrrell GJ (2000) emm and sof gene sequence variation in relation to serological typing of opacity-factor-positive group A streptococci. Microbiology 146 (Pt 5): 1195-1209.
30. Facklam RF, Martin DR, Lovgren M, Johnson DR, Efstratiou A, Thompson TA, Gowan S, Kriz P, Tyrrell GJ, Kaplan E, Beall B (2002) Extension of the Lancefield classification for group A streptococci by addition of 22 new M protein gene sequence types from clinical isolates: emm103 to emm124. Clin Infect Dis 34: 28-38.
31. Sakota V, Fry AM, Lietman TM, Facklam RR, Li Z, Beall B (2006) Genetically diverse group A streptococci from children in far-western Nepal share high genetic relatedness with isolates from other countries. J Clin Microbiol 44: 2160-2166.
32. National Committee for Clinical Laboratory Standards (2002). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically-Twelfth Informational Supplement: Approved Standard M100-S12. (NCCLS, Wayne, PA, USA.).
33. Malhotra-Kumar S, Lammens C, Piessens J, Goossens H (2005) Multiplex PCR for simultaneous detection of macrolide and tetracycline resistance determinants in streptococci. Antimicrob Agents Chemother 49: 4798-4800.
34. Smeesters PR, Campos D, Jr., Van Melderen L, de Aguiar E, Vanderpas J, Vergison A (2006) Pharyngitis in low-resources settings: a pragmatic clinical approach to reduce unnecessary antibiotic use. Pediatrics 118: e1607-1611.
35. Smeesters PR, Dramaix M, Van Melderen L The emm-type diversity does not always reflect the M protein genetic diversity--is there a case for designer vaccine against GAS. Vaccine 28: 883-885.
36. Bessen DE, Carapetis JR, Beall B, Katz R, Hibble M, Currie BJ, Collingridge T, Izzo MW, Scaramuzzino DA, Sriprakash KS (2000) Contrasting molecular epidemiology of group A streptococci causing tropical and nontropical infections of the skin and throat. J Infect Dis 182: 1109-1116.
37. Espinosa LE, Li Z, Gomez Barreto D, Calderon Jaimes E, Rodriguez RS, Sakota V, Facklam RR, Beall B (2003) M protein gene type distribution among group A streptococcal clinical isolates recovered in Mexico City, Mexico, from 1991 to 2000, and Durango, Mexico, from 1998 to 1999: overlap with type distribution within the United States. J Clin Microbiol 41: 373-378.
38. Cochetti I, Tili E, Mingoia M, Varaldo PE, Montanari MP (2008) erm(B)-carrying elements in tetracycline-resistant pneumococci and correspondence between Tn1545 and Tn6003. Antimicrob Agents Chemother 52: 1285-1290.
39. Rice LB (1998) Tn916 family conjugative transposons and dissemination of antimicrobial resistance determinants. Antimicrob Agents Chemother 42: 1871-1877.
40. Ripa S, Zampaloni C, Vitali LA, Giovanetti E, Montanari MP, Prenna M, Varaldo PE (2001) SmaI macrorestriction analysis of Italian isolates of erythromycin-resistant Streptococcus pyogenes and correlations with macrolide-resistance phenotypes. Microb Drug Resist 7: 65-71.
41. Smeesters PR, McMillan DJ, Sriprakash KS, Georgousakis MM (2009) Differences among group A streptococcus epidemiological landscapes: consequences for M protein-based vaccines? Expert Rev Vaccines 8: 1705-1720.
42. Enright MC, Spratt BG, Kalia A, Cross JH, Bessen DE (2001) Multilocus sequence typing of Streptococcus pyogenes and the relationships between emm type and clone. Infect Immun 69: 2416-2427.
43. Palavecino EL, Riedel I, Berrios X, Bajaksouzian S, Johnson D, Kaplan E, Jacobs MR (2001) Prevalence and mechanisms of macrolide resistance in Streptococcus pyogenes in Santiago, Chile. Antimicrob Agents Chemother 45: 339-341.
44. Lopardo HA, Hernandez C, Vidal P, Vazquez M, Rosaenz L, Rubinstein G, Smayevsky J, Tokumoto M, Fernandez Lausi A, Daher O, Kaufman S, Soriano SV, Brasili S, Bottiglieri M, Carranza MC (2004) Erythromycin-resistant Streptococcus pyogenes in Argentina. Medicina (B Aires) 64: 143-145.
45. Martinez S, Amoroso AM, Famiglietti A, de Mier C, Vay C, Gutkind GO (2004) Genetic and phenotypic characterization

of resistance to macrolides in Streptococcus pyogenes from Argentina. Int J Antimicrob Agents 23: 95-98.
46. Rubinstein G, Amoroso AM, Bavdaz B, De Bunder S, Blazquez N, Gutkind GO (2005) Low macrolide resistance in Streptococcus pyogenes in Southern Argentina. Int J Antimicrob Agents 25: 450-452.
47. Ayer V, Tewodros W, Manoharan A, Skariah S, Luo F, Bessen DE (2007) Tetracycline resistance in group a streptococci: emergence on a global scale and influence on multiple-drug resistance. Antimicrob Agents Chemother 51: 1865-1868.

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Conflict of interests: No conflict of interests is declared.