Genetic and phenotypic characterization of resistance to macrolides in *Streptococcus pyogenes* from Argentina

Silvia Martínez [a], Ana M. Amoroso [b], Angela Famiglietti [c], Carmen de Mier [c], Carlos Vay [c], Gabriel O. Gutkind [b],*, Working Group of Carrera de Especialización en Bacteriología [1]

[a] *Escuela de Graduados, Carrera de Especialización en Bioquímica Clínica-área Bacteriología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*
[b] *Laboratorio de Resistencia Microbiana, Cátedra de Microbiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Piso 8, 1113 Buenos Aires, Argentina*
[c] *Departamento de Bioquímica, Laboratorio de Bacteriología Clínica, Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Buenos Aires, Argentina*

Abstract

Five hundred and seventy-eight strains of group A streptococci (GAS) isolated mostly from paediatric pharyngeal swabs were tested to evaluate their susceptibility to erythromycin. Resistant strains were then tested for their MICs to erythromycin and clindamycin, their phenotype of resistance to macrolides-lincosamides-streptogramin (MLS$_B$) and for the presence of macrolide resistance genes. The rate of resistance to erythromycin was 8.2%. Constitutive, inducible and M phenotypes of resistance were detected in 2.1, 2.1 and 95.8% of resistant strains, respectively. All M phenotypes harboured the *mefA* gene, whereas constitutive and inducible phenotypes had *ermB* and *ermTR* genes, respectively.
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1. Introduction

Streptococcus pyogenes (Lancefield group A *Streptococcus*, GAS) is one of the most common human pathogens, being the responsible for the majority of cases of sore throat in paediatric patients [1]. Even after 50 years of use, penicillin remains the antibiotic of choice in the treatment of GAS infection, since *S. pyogenes* is still exquisitely sensitive to β-lactams.

In patients allergic to β-lactams, macrolides are an alternative for treatment of GAS infection. GAS resistance to erythromycin has been first described in the UK shortly after the introduction of the antibiotic into clinical practice [2]. After this and till the early 1970s, when higher rates were reported in Japan [3,4], erythromycin-resistant GAS strains were only occasionally isolated [5].

The newer erythromycin derivatives are being preferentially used for treatment of GAS pharyngitis in community medicine and empirical chemotherapy of respiratory tract infections because of their clinical efficacy, good tissue penetration and phamacokinetics, allowing less frequent dosing [6]. Meanwhile, over the past few years, increased rates of erythromycin resistance have been reported for GAS in several countries [7–17]. A positive association between macrolides use and increase in resistance was reported in Finland [18]. Furthermore, a significant reduction in the frequency of resistance was reported after an active reduction in prescription of macrolides for outpatient therapy [19].

Up to now there are two known mechanisms of macrolide resistance in GAS [20]. Methylation of 23S rRNA due to *ermB*- or *ermTR*-encoded methylase results in the inability of all macrolides, lincosamides and streptogramin B to bind to their target site in the 50 S ribosomal subunit

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* Corresponding author. Tel.: +54-11-49648285; fax: +54-11-45083645.
E-mail address: ggutkind@ffyb.uba.ar (G.O. Gutkind).

(MLS type). The methylase can be expressed constitutively (cMLS phenotype) or inducibly (iMLS phenotype). The second mechanism, giving the M phenotype, involves energy-dependent efflux of 14- and 15-membered but not 16-memebered macrolides, lincosamides and streptogramin B. The membrane-protein involved is encoded by the *mefA* gene [20–22].

The first report about GAS erythromycin resistant strains in Argentina was in 1995 [23,24]. At that time, resistant strains were only sporadically observed (1.5%). In 1997, a multicentre study involving centres all over the country was published, confirming previously reported low prevalence (1.5%) of erythromycin resistant GAS but indicating a different regional distribution of resistant strains [25]. In 2000, a regional study showed 11% resistance, a higher value than previous reports but similar to that of the former report from the same region [26]. In 2001, the latest report coming from 42 centres from the central part of the country showed an increasing 7.2% of erythromycin resistant GAS [27].

Despite resistance level surveillance in our country, there is no published report on the phenotypes and genetic mechanisms involved in GAS macrolides resistance.

The purpose of this multicentric study was to determine the susceptibilities to macrolides of GAS, and to establish the prevalent phenotype and genetic mechanism of resistance involved.

2. Materials and methods

2.1. Bacterial strains

A total of 568 non-related isolates of *S. pyogenes* recovered from eight institutions (listed in acknowledgements) were studied at the Faculty of Pharmacy and Biochemistry, University of Buenos Aires. All but one (coming from ear secretion) were from paediatric pharyngeal swabs.

Strains were identified according to Facklam [28] using bacitracin disks (0004 U) and pirrollidonil arylamidase (Laboratorios Britania, Argentina). Serology was confirmed by a commercial latex agglutination technique (Phadebact *Streptococcus* Test, Boule Diagnostics AB, Huddinge, Sweden). The isolates were stored in skimmed milk (Difco, Becton Dickinson Microbiology Systems, Spark, MD, USA) at −70 °C, and studied after being subcultured on blood agar prior to susceptibility tests.

2.2. Susceptibility testing

MICs of erythromycin and clindamycin (Sigma, St. Louis, MO) were performed by an agar dilution method according to the National Committee for Clinical Laboratory Standards guidelines [29], using Mueller Hinton agar plates supplemented with 5% sheep blood. The plates were incubated overnight at 35 °C (with 5% CO_2 if needed).

2.3. Phenotypic detection of resistance mechanisms

The resistance phenotypes of erythromycin-resistant GAS were determined by the double disk test, with erythromycin (15 μg) and clindamycin (2 μg) disks separated by 10 mm as previously described [30]. Blunting of the clindamycin inhibition zone near to the erythromycin disk indicated an inducible type of MLS_B resistance ($iMLS_B$) and resistance to both erythromycin and clindamycin indicated a constitutive type of MLS_B resistance ($cMLS_B$). Susceptibility to clindamycin with no blunting indicated the M resistance phenotype.

2.4. PCR-based detection of resistance genes

The primers used to detect *ermA*, *ermB*, *ermC*, *ermTR* and *mefA* in *S. pyogenes* were those previously described by Sutcliffe et al. [31]. DNA amplification was performed as follows: a single colony from a 24 h blood agar plate was resuspended in 20 μl of milliQ water and heated for 10 min at 100 °C in a Biometra T-Gradient thermocycler (Göttinngen, Germany). Then a mix containing 50 mM KCl, 10 mM Tris–HCl pH 9.0, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 0.2 mM deoxynucleotide triphosphate, 2 pmol of each primer and 0.6 U of *Taq* polymerase (Biotools, Madrid, Spain) was added to yield a final volume of 25 μl. Amplification was performed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 54 °C for 1 min and extension at 72 °C for 30 s, followed by an extension step at 72 °C for 10 min. Amplicons were run through 1.5% agarose gels, stained with ethidium bromide and visualized with an UV transilluminator. PCR positive controls, kindly provided by B.M. Willey (Mount Sinai Hospital, Toronto, Canada) were used for the *ermB*, *ermTR* and *mefA* genes. Erythromycin-sensitive GAS strains were used as negative PCR controls. Amplification of DNA from the positive controls with the corresponding primers yielded PCR products of the expected size: 639, 540 and 348 bp for *ermB*, *ermTR* and *mefA*, respectively [12].

3. Results and discussion

Of 568 GAS strains studied, 60 were resistant to erythromycin, with inhibition zone diameters between 6 and 14 mm. When further investigated for their erythromycin MIC by the agar dilution method, only 47 were viable, and all were erythromycin resistant (MIC > 1 mg/l).

By the double disk test, 45 isolates (95.7%) were assigned to the M phenotype, one isolate was constitutively resistant showing the $cMLS_B$ phenotype (2.1%) and another single isolate was inducibly resistant, expressing the iMLSB phenotype (2.1%).

All of the M phenotype isolates had a slightly higher than published resistance level to erythromycin (see Table 1), while clindamycin MICs values were in good agreement

Table 1
Distribution of phenotypes, macrolide susceptibility ranges and phenotypes of resistant SGA

Resistance phenotype	No. of isolates	%	Antibiotic	MIC (mg/l)			Resistance genotype
				50%	90%	Range	
M	45	95.7	Erythromycin	32	64	8–64	mefA
			Clindamycin	0.125	0.25	0.032–0.5	
cMLSB	1	2.1	Erythromycin	–	–	>128	ermB
			Clindamycin	–	–	>128	
iMLSB	1	2.1	Erythromycin	–	–	64	ermTR
			Clindamycin	0.125	0.5	0.125–0.5	

with reported data from different countries and were the same as those for the erythromycin-susceptible strains.

The *mefA* gene was present in all of the strains showing the M-resistance phenotype.

The isolate expressing a cMLS$_B$ phenotype was highly resistant to both erythromycin and clindamycin (MICs > 128 mg/l), and its characterization was confirmed genotypically by the presence of the *ermB* gene.

The inducible phenotype isolate harbouring the *ermTR* gene (now considered a variant of *ermA*) [32,33], had MIC values similar to those reported previously.

Genes coding for both resistance mechanisms were not found in the same SGA strain. No amplification was detected in any of the strains when primers specific for *ermA* or *ermC* were used in PCR.

Of the Argentinean GAS isolates studied, 8.27% were resistant to erythromycin using the agar dilution method. This means that, even if this value remains relatively low when compared with those from others countries, in the last 5 years, erythromycin resistance rates have increased five-fold in our country.

The predominant phenotype was M and was present in more than 95% of analyzed erythromycin-resistant GAS. The high incidence of the M-resistant phenotype found in this study agrees with published results from others groups indicating that the efflux pump MefA-resistance mediated mechanism is also predominant in Europe.

When compared with some European countries, our resistance rate remains relatively low, and hopefully, stabilized, suggesting that erythromycin and clindamycin remain as possible alternatives for the treatment of SGA infections. Careful usage of macrolide antibiotics and continued surveillance of resistance rate is advisable.

Acknowledgements

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