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In Vitro Activity of Solithromycin against Erythromycin-Resistant *Streptococcus agalactiae*

Giorgio Piccinelli,[a] Prabhavathi Fernandes,[b] Carlo Bonfanti,[a] Francesca Caccuri,[a] Arnaldo Caruso,[a] Maria Antonia De Francesco[a]

Institute of Microbiology, Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy[a]; Cempra, Inc., Chapel Hill, North Carolina, USA[b]

The *in vitro* antibacterial activity of solithromycin (CEM-101) against macrolide-resistant isolates ($n = 62$) of *Streptococcus agalactiae* (group B streptococcus [GBS]) was determined. Phenotypic characterization of macrolide-resistant strains was performed by double-disc diffusion testing. A multiplex PCR was used to identify the *erm*(B), *erm*(TR), and *mef*(A/E) genes, capsular genotypes, and alpha-like (Alp) protein genes from the GBS strains. Determination of MIC was carried out using the microdilution broth method. The Etest method was used for penicillin, azithromycin, clarithromycin, and erythromycin. Solithromycin had a MIC_{50} of ≤0.008 μg/ml and a MIC_{90} of 0.015 μg/ml against macrolide-susceptible *S. agalactiae*. These MICs were lower than those displayed by penicillin (MIC_{50} of 0.032 μg/ml and MIC_{90} of 0.047 μg/ml), the antibiotic agent of choice for prophylaxis and treatment of GBS infections. Against macrolide-resistant *S. agalactiae*, solithromycin had a MIC_{50} of 0.03 μg/ml and a MIC_{90} of 0.125 μg/ml. Against *erm*(B) strains, solithromycin had a MIC_{50} of 0.03 μg/ml and a MIC_{90} of 0.06 μg/ml, while against *mef*(A) strains, it had a MIC_{50} of 0.03 μg/ml and a MIC_{90} of 0.125 μg/ml. Most erythromycin-resistant GBS strains were of serotype V (64.5%) and associated significantly with *alp2-3*. Moreover, a statistically significant association was observed between the constitutive macrolide-lincosamide-streptogramin B resistance ($cMLS_B$) phenotype and the *erm*(B) gene-carrying strains, the *alp2-3* gene and the M phenotype, and the *mef*(A/E) gene and *epsilon*. Overall, our results show that solithromycin had lower or similar MICs than penicillin and potent activity against macrolide-resistant strains independent of their genotype or phenotype, representing a valid therapeutic alternative where β-lactams cannot be used.

Streptococcus agalactiae (group B streptococcus [GBS]) is a common cause of severe infections in neonates, such as sepsis and meningitis. It is also an important pathogen causing bacteremia and endocarditis in elderly patients, patients with diabetes, and immunocompromised subjects (1, 2). The highest GBS mortality and morbidity result from invasive infections in neonates, particularly in those with very low birth weight (3, 4). Due to the severity of disease resulting from *S. agalactiae* infections in neonates, the elderly, diabetics, and immunocompromised patients, the U.S. Food and Drug Administration (FDA) has recently proposed *S. agalactiae* as a qualified infectious diseases pathogen (5).

Penicillin is the first-line antibiotic for treatment of GBS infection, as well as for intrapartum antibiotic prophylaxis to prevent early-onset infection, because resistance to this agent has not been reported so far among GBS clinical isolates. Macrolides are the recommended second-line drugs and the first alternative in cases of β-lactam allergy.

However, in 2008, GBS clinical isolates were identified with reduced penicillin susceptibility, in which an increase was observed in the MICs of β-lactam antibiotics, including penicillin (MICs of 0.25 to 1 mg/liter) (6, 7). In addition, the rates of erythromycin resistance have increased at different levels in various regions in the world (8, 9). There are two mechanisms of resistance to macrolides: one is a modification of the ribosomal target site by a dimethylation of an adenine residue in the 23S rRNA, encoded by *erm* genes, and the other involving increased efflux of the drug outside the organism by macrolide efflux pumps, encoded by *mef* genes. Target site modification confers inducible ($iMLS_B$) or constitutive ($cMLS_B$) resistance to all antibiotics in the macrolide-lincosamide-streptogramin B group, while the presence of the efflux pump confers resistance only to 14- and 15-membered macrolides (M phenotype).

To overcome the macrolide resistance of Gram-positive cocci, the ketolides, which are macrolide analogs, were developed to treat respiratory infections due to microorganisms (*Streptococcus pneumoniae* and *Streptococcus pyogenes*) that are macrolide resistant.

Telithromycin was the first ketolide introduced as the drug able to address the macrolide resistance problem and received FDA approval in 2004. However, because of severe adverse events (10, 11), it is approved for use only in community-acquired bacterial pneumonia (CABP).

Solithromycin (CEM-101) is a novel fluoroketolide that shows activity comparable or superior to those of telithromycin, azithromycin, erythromycin, and clarithromycin, with high potency against Gram-positive and Gram-negative bacteria, as well as activity against most macrolide-resistant bacteria (12–14). It is currently being evaluated in a phase 3 trial as monotherapy for CABP.

The aim of this study was to evaluate the *in vitro* activity of solithromycin against a spectrum of *S. agalactiae* strains with different macrolide resistance genotypes and phenotypes compared to those of penicillin G, erythromycin, azithromycin, and clarithromycin. This collection of strains was further characterized for surface proteins and capsular type, which represent important virulence factors of GBS.

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Address correspondence to Maria Antonia De Francesco, defrance@med.unibs.it.

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TABLE 1 Activities of solithromycin and comparator antimicrobial agents against *Streptococcus agalactiae*

Organism	Antimicrobial drug	MIC (mg/liter)			
		50%	90%	Range observed	Range tested
Erythromycin-resistant GBS ($n = 62$)	Solithromycin	0.03	0.125	≤0.008–1	0.008–4
	Penicillin	0.032	0.047	0.012–0.06	0.002–32
	Erythromycin[a]	>8	>8	0.5->8	0.25–8
	Erythromycin[b]	>256	>256	0.5->256	0.016–256
	Azithromycin	>256	>256	0.19->256	0.016–256
	Clarithromycin	>256	>256	0.25->256	0.016–256
Erythromycin-susceptible GBS ($n = 10$)	Solithromycin	≤0.008	0.015	≤0.008–0.03	0.008–4
	Penicillin	0.032	0.047	0.012–0.047	0.002–32
	Erythromycin[a]	≤0.25	≤0.25	≤0.25	0.25–8
	Erythromycin[b]	0.047	0.047	0.012–0.064	0.016–256
	Azithromycin	≤0.125	≤0.125	0.019–0.19	0.016–256
	Clarithromycin	0.047	0.047	0.023–0.047	0.016–256

[a] Broth microdilution test.
[b] Etest.

MATERIALS AND METHODS

Strain collection. A total of 72 clinical isolates of *S. agalactiae*, which had been collected from Brescia's main hospital (Spedali Civili) between 2005 and 2012, were used in the MIC determination study. The isolates were recovered from different specimens (23 urine samples, 43 vaginal samples, 3 urethral swabs, and 3 rectal swabs). GBS strains were isolated by streak plating 1 to 10 μl of transport medium on ChromID streptoB agar plate (bioMérieux, St. Louis, MO). The plates were incubated at 37°C for 18 to 24 h under aerobic conditions. GBS was selected by the production of a pink pigment when grown aerobically on ChromID streptoB agar. GBS identification was performed by means of the Vitek system (bioMérieux).

Capsular gene typing. The capsular genotype (Ia, Ib, and II to IX) of *S. agalactiae* was identified by a multiplex PCR assay as previously described (15). DNA was extracted from each strain using a DNeasy kit (Qiagen). Approximately 1 ng of DNA was used in the PCRs with primers and conditions as described elsewhere (15). Serotypes of strains were identified by analyzing the unique banding pattern following 1.5% (wt/vol) agarose gel electrophoresis.

Alp genes. The alpha-like protein (Alp) genes *bca*, *alp1* (*Epsilon*), *alp2/3*, *Rib*, and *alp4* in the strains were detected by using a multiplex PCR as previously described (16). In brief, the PCR mixture (total volume, 25 μl) contained 1 ng of DNA template, 1× PCR buffer, 2 mmol/liter of $MgCl_2$, 200 μmol/liter of deoxynucleoside triphosphates (dNTPs), 400 nmol/l of each of the five pairs of primers, and 0.3 U of AmpliTaq Gold (Roche). Amplification conditions were as previously described (16). Amplification of the alpha-like protein genes was evaluated by agarose gel (2%, wt/vol) electrophoresis of the PCR products.

Antimicrobial resistance phenotype and genotype. Phenotypic characterization of macrolide-resistant strains was performed by double-disc diffusion testing as described previously (17). Erythromycin (15 μg) and clindamycin (2 μg) discs were placed 20 mm apart. Isolates resistant to erythromycin with blunting of the clindamycin inhibition were of the $iMLS_B$ phenotype, isolates that demonstrated resistance to both erythromycin and clindamycin were of the $cMLS_B$ phenotype, isolates showing resistance to erythromycin without blunting of the clindamycin inhibition zone were of the M phenotype, and isolates resistant to clindamycin yet susceptible or intermediate to erythromycin belonged to the L phenotype. Interpretive criteria were according to CLSI guidelines (18). A multiplex PCR was used to identify the *erm*(B), *erm*(TR), and *mef*(A/E) genes from the GBS strains, using primers and conditions previously reported (17–19), and a separate PCR was used to amplify the *lin*(B) gene (20, 21).

Antimicrobial agents and MIC determination. Solithromycin (CEM-101) was obtained from Cempra, Inc., Chapel Hill, NC. Determination of MIC was carried out using the microdilution broth method according to CLSI guidelines (22). In brief, an inoculum of approximately 5×10^5 to 5×10^6 CFU/ml was incubated with a concentration of solithromycin ranging from 0.008 to 4 μg/ml. *S. pneumoniae* ATCC 49619 was used as a quality control. Results were observed after 18 h of incubation at 37°C. For comparison to solithromycin, penicillin, azithromycin, clarithromycin, and erythromycin were used. The Etest method (Liofilchem, Italy) was used for all of the reference antibiotics. The test was performed according to the manufacturer's instructions. Antibiotic concentrations ranged from 0.002 to 32 μg/ml for penicillin and from 0.016 to 256 μg/ml for azithromycin, clarithromycin, and erythromycin. Erythromycin was also tested by an automated microdilution broth method (Vitek2; bioMérieux). The concentrations ranged from 0.25 to 8 μg/ml. Breakpoint interpretation was done according to EUCAST guidelines (23), and breakpoints were as follows: penicillin, ≤0.25 and >0.25 μg/ml, susceptible and resistant, respectively; erythromycin, azithromycin, and clarithromycin, ≤0.25 and >0.5 μg/ml, susceptible and resistant, respectively.

Statistical analysis. The χ^2 test was used to evaluate the differences in distributions of surface proteins, serotypes, genotypes and phenotypes. A P value of <0.05 was considered significant, and a P value of <0.01 was considered highly significant.

RESULTS

MICs of antimicrobial agents for clinical strains. The activities of solithromycin and the comparator antimicrobial agents against clinical strains are shown in Table 1. The MIC_{50} and the MIC_{90} of solithromycin were ≤0.008 and 0.015 μg/ml against erythromycin-susceptible strains, which were respectfully at least 4-fold and 3-fold lower than that of penicillin, the first-line agent both for intrapartum antibiotic prophylaxis and for the treatment of GBS infections in adults. On the other hand, erythromycin and clarithromycin had a MIC_{50} and a MIC_{90} comparable to that of penicillin, while azithromycin had both a MIC_{50} and MIC_{90} of ≤0.125 μg/ml. Against erythromycin-resistant strains, solithromycin had a MIC_{50} of 0.03 μg/ml and a MIC_{90} of 0.125 μg/ml. The MIC_{50} of penicillin was 0.032 and comparable to that of solithromycin, whereas the MIC_{90} of penicillin was 2.7-fold lower than that of solithromycin against erythromycin-resistant strains.

Evaluation of macrolide-resistant genotypes and phenotypes of GBS. The determination of macrolide-resistant genotypes in GBS was performed to evaluate the differences in the

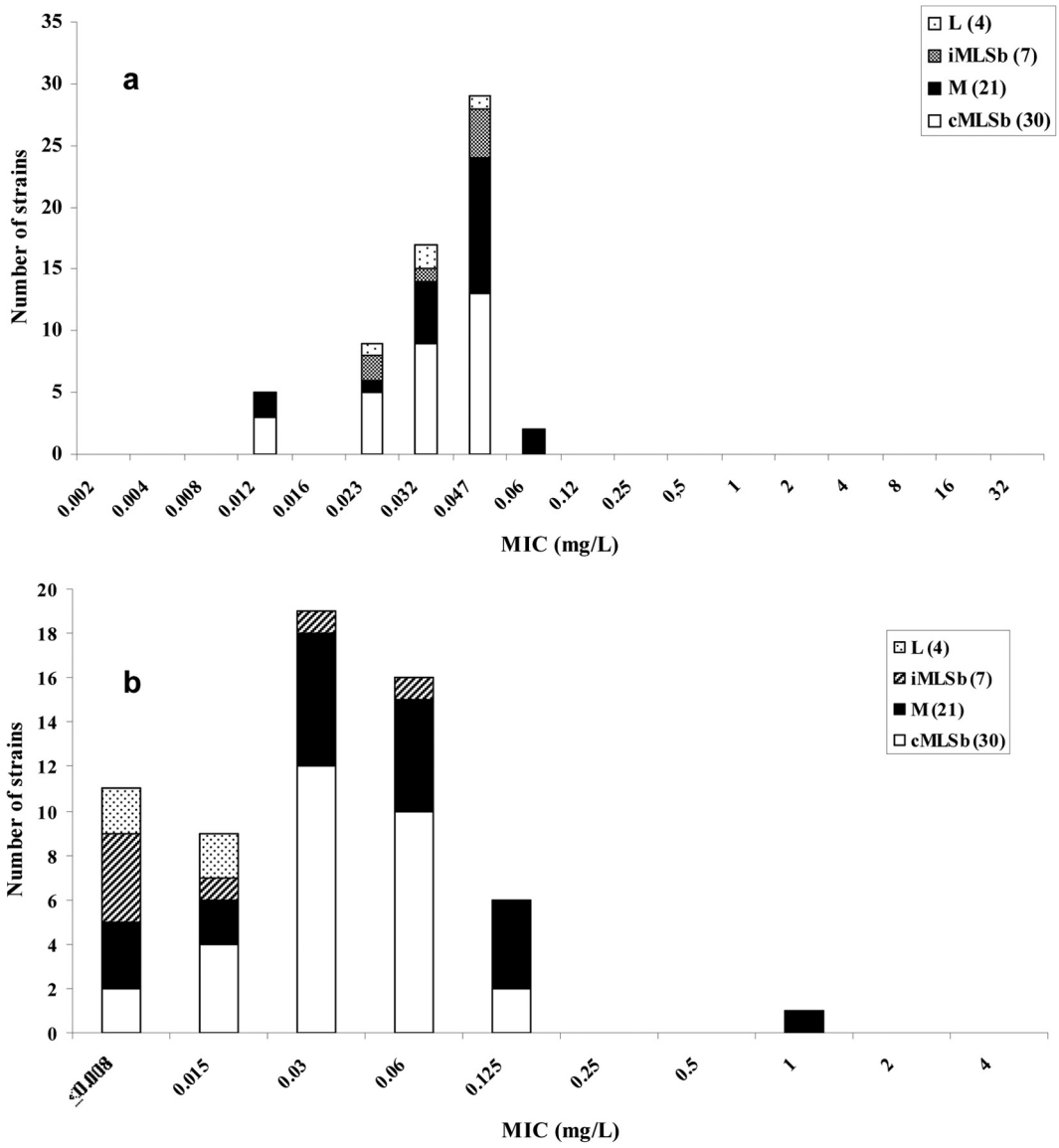

FIG 1 MIC distribution of penicillin (a) and solithromycin (b) for the different phenotypes of macrolide-resistant *Streptococcus agalactiae* strains.

activities between solithromycin and the other antimicrobial agents tested. Among the 62 macrolide-resistant clinical strains, 30 displayed the $cMLS_B$ phenotype, 21 the M phenotype, 7 the $iMLS_B$ phenotype, and 4 the L phenotype. Regarding L phenotypes, three were erythromycin-intermediate and clindamycin-resistant strains and one was erythromycin susceptible and clindamycin resistant by the disc diffusion test. To identify the cause of macrolide resistance, we screened for the presence of several genes. Most of the screened strains possessed a single resistance gene. Among these strains, the *erm*(B) gene was present in 26 strains and was mostly associated with the $cMLS_B$ phenotype, with a MIC of >256 μg/ml for almost all of the reference macrolides. The *mef*(A/E) gene was present in 22 strains, while *erm*(A) [subclass *erm*(TR)] was identified in 3 strains. The *lin*(B) gene was not detected in any GBS strains, and the L phenotypes observed were associated with the *erm*(B) gene (3 strains) and the *mef*(A/E) gene (1 strain). Eleven strains possessed more than one resistance gene. There were five isolates with a susceptible phenotype in which the presence of a resistance gene was detected, including the *erm*(B) gene (2 isolates), the *erm*(A) [subclass *erm*(TR)] (2 isolates), and one isolate that had both *erm*(B) and *erm*(A) [subclass *erm*(TR)].

Activities of the different antimicrobial agents against the various macrolide-resistant genotypes and phenotypes of GBS. MIC distributions of solithromycin and penicillin for the different phenotypes of GBS are shown in Fig. 1. For solithromycin, most of the strains that displayed the $cMLS_B$ phenotype had a MIC between 0.03 and 0.06 μg/ml, while for penicillin the MIC range was between 0.03 and 0.047 μg/ml. Similar MIC distributions were observed for strains with the M phenotype. In contrast, most of the strains that had the $iMLS_B$ phenotype had a MIC of 0.047 μg/ml for penicillin and a MIC of ≤0.008 μg/ml for solithromycin.

Strains with the L phenotype had a MIC distribution between

TABLE 2 MIC_{50}s and MIC_{90}s of solithromycin and comparator drugs against *S. agalactiae* strains with defined macrolide-resistant genotypes

Drug	MIC (mg/liter) for strains with different macrolide resistance mechanisms (no. of strains)			
	erm(B) (26)		*mef*(A/E) (22)	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Solithromycin	0.03	0.06	0.03	0.125
Penicillin	0.032	0.047	0.047	0.047
Erythromycin[a]	>8	>8	>8	>8
Erythromycin[b]	>256	>256	6	>256
Azithromycin	>256	>256	12	>256
Clarithromycin	>256	>256	6	>256

[a] Broth microdilution test.
[b] Etest.

TABLE 3 Distribution of the *alp* genes among the observed GBS serotypes

Surface alpha-like protein gene (no. of isolates)	No. of isolates per serotype[a]						
	Ia	Ib	II	III	IV	V	VI
alp2-3 (30)	2			3		25**	
rib (33)	4	3	3	9*	2	12	
epsilon (22)	6*				2	14	
alpha c (10)	2			2		6	
alp 4 (14)	1			4		8	1

[a] *, $P < 0.05$; **, $P < 0.01$.

≤0.008 and 0.015 μg/ml for solithromycin and between 0.023 and 0.047 μg/ml for penicillin (Fig. 1).

The majority of the *erm*(B) gene-carrying strains of *S. agalactiae* showed high resistance (MIC > 256 μg/ml) to clarithromycin and azithromycin. In contrast, solithromycin showed a MIC_{90} of 0.06 μg/ml against the same strains (Table 2). The MIC_{90} of solithromycin against *mef*(A/E) strains was 0.125 μg/ml, whereas the MIC_{90} for *erm*(B) gene-carrying strains was 2-fold lower (0.06 μg/ml).

Capsule serotyping and the *alp* family genes. The detection of genes encoding particular capsular serotypes was performed by multiplex PCR. Overall, the most represented serotypes isolated were types V ($n = 40$ [55.5%]), III ($n = 12$ [16.6%]), and Ia ($n = 10$ [13.8%]), followed by serotypes Ib, II, and IV, which were represented by three isolates each (4%). One strain belonged to serotype VI. No strains of serotypes VII and VIII were found in this pool of isolates.

Surface proteins of GBS are likely to play an important role in the pathogenesis of *S. agalactiae* infection; therefore, they were evaluated by PCR. The presence of a particular *alp* gene in relation to the serotype was noted (Table 3).

Of the 30 *alp2-3*-positive strains isolated, 25 were of serotype V; 14 of 22 *epsilon*-positive strains corresponded to serotype V, and 6 corresponded to serotype Ia. *rib*-positive strains were present in almost all serotypes isolated. Conversely, a certain serotype commonly corresponded to a particular Alp gene: serotype Ib and II presented *rib*, serotype IV carried either *rib* or *epsilon*, many serotype V strains (62.5%) possessed *alp2-3*, and serotype Ia predominantly carried *epsilon* (6/15 isolates), while *rib* was the most common surface protein associated with the serotype III (9/18) ($P < 0.05$). Different associations of *alp* genes were present in a single strain. Regarding the phenotypes and genotypes, we found a statistically significant association between the $cMLS_B$ phenotype and the *erm*(B) gene-carrying strains ($P < 0.05$), between the $cMLS_B$ phenotype and *alp2-3* ($P < 0.05$), between the M phenotype and *epsilon* ($P < 0.05$), and between the *mef*(A/E) gene-carrying strains and *epsilon* ($P < 0.001$) (data not shown).

DISCUSSION

The recent emergence of *S. agalactiae* strains with reduced penicillin susceptibility in Japan and the United States constitutes a problem for the use of this drug in prophylaxis (6, 24). The increasing importance of *S. agalactiae* has been noted by its inclusion in the list of proposed qualified pathogens by the FDA. The molecular analysis of these particular strains showed a mutagenic pathway comparable to that observed when the first β-lactam-resistant *S. pneumoniae* strains were isolated. The emergence of a physiologically GBS pbp2x (Q557E) mutant is worrying, because the accumulation of additional mutations might lead to complete penicillin resistance. This suggests a potential risk of therapeutic failure of intrapartum prophylaxis in the near future.

Traditionally the macrolides, and in particular erythromycin, have been considered the second-line choice of antibiotic in patients allergic to β-lactams. However, resistance to macrolides and lincosamides has risen during the last decades, with 19% of the *S. agalactiae* isolates resistant to erythromycin and 53% of these showing resistance to clindamycin (25). Regarding the erythromycin resistance among strains of *S. agalactiae*, we have previously found a resistance rate of 15% (26), a result similar to what has been observed in Spain, Portugal, Germany, France, and Canada (27–30), but resistance rates differ considerably between regions, with a rate of only 3.8% reported in the Czech Republic (31) and 38% to 41.9% in the United States (8).

To address the resistance problem, new macrolide antibiotics called ketolides have been developed that have potent activity against erythromycin-resistant streptococci.

In the collection of *S. agalactiae* isolates used in this study, there was a predominance of $cMLS_B$ and M phenotypes, indicating that erythromycin resistance was mediated by the two principal mechanisms: methylation of 23S rRNA, determined by *erm* genes, and active drug efflux by pumps encoded by *mef* genes. These strains showed cross-resistance to clarithromycin and azithromycin, with MIC_{90}s of >256 μg/ml.

The novel fluoroketolide solithromycin tested in this study demonstrated superior potency over older macrolides against all macrolide-resistant strains, with a MIC_{90} of 0.125 μg/ml. The enhanced activity of solithromycin over other ketolide compounds is likely due to a higher binding affinity to bacterial ribosomes based on an 11,12-carbamate-butyl-[1,2,3]-triazolyl-amino-phenyl side chain as well as a 2-fluoro modification (32). Solithromycin demonstrated potent activity against macrolide-susceptible GBS, with a MIC_{90} of 0.015 μg/ml, which was 3-fold lower than that of penicillin. Although strains with either *mef*(A/E) or *erm*(B) have slightly higher solithromycin MICs than susceptible strains, the solithromycin MIC for macrolide-resistant GBS rarely exceeds 0.125 μg/ml. This lower MIC suggests that this drug may be useful in the treatment of infections caused by these pathogens. There were five isolates of the $cMLS_B$ phenotype that had both *erm*(A) subclass *erm*(TR) and *erm*(B) genes; the coexistence of both genes has been documented previously (33). Furthermore, three isolates

that displayed the $cMLS_B$ phenotype harbored both *mef*(A/E) genes and *erm*(B), and one isolate that had the $iMLS_B$ phenotype had both *erm*(A) subclass *erm*(TR) and *mef*(A/E). This finding implies differential gene expression, as only the *erm*(B) gene and *erm*(TR) gene were expressed in the different isolates, respectively. Exceptionally and for the first time, to our knowledge, we found one strain that harbored all three macrolide resistance genes and displayed the $cMLS_B$ phenotype.

We observed that all of the GBS strains that had the $iMLS_B$ phenotype and harbored the *erm*(B) or the *erm*(A) (subclass *erm*TR) gene expressed low-level resistance to erythromycin (MICs, 1 to 12 μg/ml) but high azithromycin MICs in absolute terms (2 to >256 μg/ml). This unusual resistance pattern has been previously identified in macrolide-resistant *S. pyogenes* strains harboring the *erm*(A) gene with point mutations in the *erm*(A) regulatory region leading to constitutive methylase expression (34). Whether or not this was the case for the strains isolated in this study requires further evaluation.

Further, we identified five macrolide-susceptible strains that contained the *erm*(B) or *erm*(TR) gene or both, as has been reported previously (17). Whether it is possible for these susceptible strains carrying macrolide resistance genes to become resistant upon environmental stimulus or over time is unknown.

It has been hypothesized previously that the spread of strains of particular surface protein profiles and serotypes reflects the selection of the best evolutionary lineages by the immune system (35). In this study, we found that our isolates presented serotype-surface protein gene combinations (serotype V-*alp2-3* and serotype III-*rib*) already reported (35, 36) and a different combination (serotype Ia-*epsilon*) that we observed in a previous study (26), suggesting that new successfully selected clones may be emerging. Moreover, statistically significant associations were observed between the $cMLS_B$ phenotype and the *erm*(B) gene-carrying strains, *alp2-3* and the M phenotype, and the *mef* (A/E) gene-carrying strains and *epsilon*.

Among strains resistant to macrolides, the V serotype dominated (40/62 [64.5%]), an association previously reported (37). Our results are consistent with the literature and underline the spread of a phenomenon during the past years, which is an increasing number of GBS isolates being resistant to erythromycin, representing serotype V. Given this trend, the excellent activity of solithromycin against macrolide-susceptible and macrolide-resistant GBS observed in this study becomes more relevant, as this compound may represent a valid alternative in the treatment of infections caused by this pathogen, in particular if there is a limitation of therapeutic options.

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