In vitro activity of solithromycin against erythromycin-resistant Streptococcus agalactiae

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ABSTRACT

In vitro antibacterial activity of solithromycin (CEM-101) against macrolide-resistant (n=62) *Streptococcus agalactiae* (GBS) was determined. Phenotypic characterization of macrolide-resistant strains was performed by double-disc diffusion testing. A multiplex PCR was used to identify the *ermB*, *ermTR*, and *mefA/E* genes, capsular genotypes and alpha-like (Alp) protein genes from the GBS strains. Determination of minimal inhibitory concentration (MIC) was carried out using the microdilution broth method. Etest method was used for penicillin, azithromycin, clarithromycin and erythromycin.

Solithromycin had a MIC<sub>50</sub> of ≤0.008 μg/ml and a MIC<sub>90</sub> of 0.015 μg/ml against macrolide-susceptible *S. agalactiae*. These MICs were lower than those displayed by penicillin (MIC<sub>50</sub> of 0.032 μg/ml and MIC<sub>90</sub> 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<sub>50</sub> of 0.03 μg/ml and a MIC<sub>90</sub> of 0.125 μg/ml. Against *ermB* strains, solithromycin had a MIC<sub>50</sub> of 0.03 μg/ml and a MIC<sub>90</sub> of 0.06 μg/ml, while against *mefA* strains it had a MIC<sub>50</sub> of 0.03 μg/ml and a MIC<sub>90</sub> 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 cMLS<sub>B</sub> phenotype and the *ermB* 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 compared to 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.
INTRODUCTION

*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 in 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, elderly, diabetics, and immunocompromised patients, the United States Food and Drug Administration has recently proposed *S. agalactiae* as a Qualified Infectious Diseases Pathogen (Federal Register. Establishing a List of Qualifying Pathogens Under the Food and Drug Administration Safety and Innovation Act. A Proposed Rule by the Food and Drug Administration on 06/12/2013).

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 beta-lactam antibiotics including penicillin (MICs of 0.25-1 mg/L) (5, 6). In addition, the rates of erythromycin resistance have increased at different levels in various regions in the world (7, 8). 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 (iMLSb) or constitutive (cMLSb) 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, 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 (9, 10), it is only approved for use in community-acquired bacterial pneumoniae (CABP).

Solithromycin (CEM-101) is a novel fluoroketolide that shows activity comparable or superior to telithromycin, azithromycin, erythromycin and clarithromycin with high potency against Gram-positive and Gram-negative bacteria, as well as activity against most macrolide-resistant bacteria (11-13). 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.
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, USA). The plates were incubated at 37°C for 18 to 24h in 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, II–IX of *S. agalactiae* was identified by a multiplex PCR assay as previously described (14). 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 (14). Serotypes of strains were identified by analyzing the unique banding pattern following agarose gel 1.5% (wt/vol) electrophoresis.

Alpha-like protein (Alp) genes

The alpha-like protein genes *bca*, *alp1* (*Epsilon*), *alp2/3*, *Rib*, and *alp4* in the strains were detected by using a multiplex PCR as already described (15). In brief, the PCR mix (total volume, 25μl) contained: 1ng DNA template, 1x PCR buffer, 2mmol/l MgCl₂, 200 μmol/l dNTPs, 400 nmol/l of each of the five pairs of primers, and 0.3 U of AmpliTaq Gold (Roche). Amplification conditions were performed as already described (15). 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 (16). 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\textsubscript{B} phenotype, isolates that demonstrated resistance to both erythromycin and 
clindamycin were of the cMLS\textsubscript{B} phenotypes, 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.

Interpretative criteria were according to CLSI guidelines (17). A multiplex PCR was used to 
identify the \textit{erm}(B), \textit{erm}(TR), and \textit{mef}(A/E) genes from the GBS strains, using primers and 
conditions previously reported (16-18) and a separate PCR was used to amplify the \textit{lin}(B) gene (19, 
20).

\textbf{Antimicrobial agents and MIC determination}

Solithromycin (CEM-101) was obtained from Cempra, Inc., Chapel Hill, NC. Determination of 
minimal inhibitory concentration (MIC) was carried out using the microdilution broth method 
according to CLSI guidelines (21). In brief, an inoculum of approximately $5 \times 10^5$-5 $\times 10^6$ CFU/ml 
was incubated with a concentration of solithromycin ranging from 0.008 to 4 $\mu$g/ml. \textit{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 $\mu$g/ml for penicillin and from 0.016 to 256 $\mu$g/ml for azithromycin, clarithromycin and 
erthyromycin. Erythromycin was also tested by an automated microdilution broth method (Vitek2, 
Biomérieux). The concentrations ranged from 0.25 to 8 $\mu$g/ml. Breakpoint interpretation was done 
according to EUCAST guidelines (22) and it was as follows: penicillin, $\leq 0.25$ and $>0.25$ $\mu$g/ml, 
susceptible and resistant, respectively; erythromycin, azithromycin and clarithromycin, $\leq 0.25$ and 
$>0.5$ $\mu$g/ml, susceptible and resistant, respectively.
Statistical analysis

The χ2 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\textsubscript{50} and the MIC\textsubscript{90} of solithromycin were \( \leq 0.008 \) and \( 0.015 \) \( \mu \)g/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\textsubscript{50} and a MIC\textsubscript{90} comparable to that of penicillin, while azithromycin had both a MIC\textsubscript{50} and MIC\textsubscript{90} of \( \leq 0.125 \) \( \mu \)g/ml. Against erythromycin-resistant strains, solithromycin had a MIC\textsubscript{50} of 0.03 \( \mu \)g/ml and a MIC\textsubscript{90} of 0.125 \( \mu \)g/ml. The MIC\textsubscript{50} of penicillin was 0.032 and comparable to that of solithromycin, whereas the MIC\textsubscript{90} of penicillin was 2.7-fold lower than 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 activities between solithromycin and the other antimicrobial agents tested. Among the 62 macrolide-resistant clinical strains, thirty displayed the cMLS\textsubscript{B} phenotype, twenty-one the M phenotype, seven the inducible iMLS\textsubscript{B} phenotype and four the L phenotype. Regarding L phenotypes, three were erythromycin-intermediate and clindamycin-resistant strains and one was erythromycin-susceptible and clindamycin resistant strain by disk 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 \textit{erm}(B) gene was present in 26 strains and it was mostly associated to the cMLS\textsubscript{B} phenotype, with a MIC of >256 \( \mu \)g/ml for almost all of the reference macrolides. The \textit{mef}(A/E) gene was present in 22 strains, while \textit{erm}(A) (subclass \textit{ermTR}) was identified in 3 strains. The \textit{lin}(B) gene was not detected in any GBS strains and the L phenotypes observed were associated with the \textit{erm}(B) gene (3 strains) and the \textit{mef}(A/E) gene (1 strain). Eleven strains possessed more than one resistance gene. There were five isolates
with a susceptible phenotype where the presence of a resistance gene was detected including the 
$erm(B)$ gene (2 isolates), the $erm(A)$ (subclass $ermTR$) (2 isolates), and one isolate that had both the 
$erm(B)$ and $erm(A)$ (subclass $ermTR$).

Activity 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 Figure 1. For solithromycin, most of the strains that displayed the cMLS$_B$ phenotype had a MIC between 0.03 and 0.06 $\mu$g/ml, while for penicillin the MIC range was between 0.03 and 0.047 $\mu$g/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 $\mu$g/ml for penicillin and a MIC of 
$\leq$0.008 $\mu$g/ml for solithromycin.

Strains with the L phenotype had a MIC distribution between $\leq$0.008 and 0.015 $\mu$g/ml for 
solithromycin and between 0.023 and 0.047 $\mu$g/ml for penicillin (Figure 1).

The majority of the $erm(B)$ gene carrying strains of $S. agalactiae$ showed high resistance (MIC>256 
$\mu$g/ml) to clarithromycin and azithromycin. In contrast, solithromycin showed a MIC$_{90}$ of 
0.06 $\mu$g/ml against these same strains (Table 2). The MIC$_{90}$ of solithromycin against $mef(A/E)$ 
strains was 0.125 $\mu$g/ml, whereas the MIC$_{90}$ for $ermB$ gene carrying strains was two-fold lower 
(0.06 $\mu$g/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 that were represented by three isolates each 
(4%). One strain belonged to the 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 *alp*2-3-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 *alp*2-3; serotype Ia predominately 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 the *alp*2-3 (*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 USA constitutes a problem for the use of this drug in prophylaxis (23, 5). The increasing importance of *S. agalactiae* has been noted by its inclusion in the list of proposed qualified pathogens by the United States Food and Drug Administration. The molecular analysis of these particular strains showed a mutagenic pathway comparable to that observed when the first beta-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 a 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 that showed resistance to clindamycin (24). Regarding the erythromycin resistance among strains of *S. agalactiae*, we have previously found a resistance rate of 15% (25), a result similar to what has been observed in Spain, Portugal, Germany, France and Canada (26-29), but resistance rates differ considerably between regions with a rate of only 3.8% reported in Czech Republic (30) and 38%-41.9% in the United States (7).

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

In this collection of *S. agalactiae* isolates, there was a predominance of cMLS_B and M phenotypes indicating that erythromycin resistance was mediated by the two principal mechanisms: methylation of 23S ribosomal RNA, determined by *erm* genes, and active drug efflux by pumps encoded by *mef* genes. These strains showed cross-resistance to clarithromycin and azithromycin, with MIC₉₀'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 MIC₉₀ 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 (31). Solithromycin demonstrated potent activity with a MIC₉₀ of 0.015 μg/ml for against macrolide-susceptible GBS, which was three-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. These lower MICs suggest that this drug may be useful in the treatment of infections caused by these pathogens. There were five isolates of the cMLS₉ phenotype that had both erm(A) subclass erm(TR) and erm(B) genes; the coexistence of both genes has been documented previously (32). Furthermore, three isolates that displayed cMLS₉ phenotypes harboured both mef(A/E) genes as well as erm(B) genes and one isolate that had the iMLS₉ phenotype had both erm(A) subclass erm(TR) and mef(A/E) genes. 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 harboured all three macrolide resistance genes and displayed the cMLS₉ phenotype.

We observed that all of the GBS strains that had the iMLS₉ phenotype and harboured the erm(B) or the erm(A) (subclass erm(TR)) genes expressed low-level resistance to erythromycin (MICs 1-12 μg/ml), but high azithromycin MICs in absolute terms (2 - >256μg/ml). This unusual resistance pattern has been previously identified in macrolide-resistant S. pyogenes strains harbouring the erm(A) gene with point mutations in the erm(A) regulatory region leading to constitutive methylase expression (33). 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) genes or both, as has been reported previously (16). 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 (34). In this study we found that our isolates presented serotype-surface protein gene combinations (serotype V-\textit{alp}2,3; serotype III-\textit{rib}) already reported (34, 35) and a different combination (serotype Ia-\textit{epsilon}) that we observed in a previous study (25), suggesting that new successfully selected clones may be emerging. Moreover, a statistically significant association was observed between the c\textit{MLS}B phenotype and the \textit{erm}(B) gene carrying strains, and the \textit{alp}2-3 and the M phenotype, and the \textit{mef} (A/E) gene carrying strains and \textit{epsilon}. Among strains resistant to macrolides, the V serotype dominated (40/62, 64.5%), an association already reported (36). 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.
ACKNOWLEDGMENTS

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We thank Kara Keedy for her assistance in the preparation of the manuscript.
References


17. Clinical and Laboratory Standards Institute. Performance standard for antimicrobial susceptibility testing, M100-S20 (2010), M-2 and M-7, Wayne, PA


22. EUCAST. Breakpoint tables for interpretation of MICs and zone diameters Version2.0, January 2012.


Fig. 1. MIC distribution of penicillin (a) and solithromycin (b) for the different phenotypes of macrolide-resistant Streptococcus agalactiae strains.
Table 1. Activity of solithromycin and comparator antimicrobial agents against *Streptococcus agalactiae* (GBS)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antimicrobial drug</th>
<th>MIC (mg/L)</th>
<th>50%</th>
<th>90%</th>
<th>Range Observed</th>
<th>Range Tested</th>
</tr>
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<tr>
<td><strong>Erythromycin-resistant GBS (n=62)</strong></td>
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</tr>
<tr>
<td></td>
<td>Solithromycin</td>
<td>0.03</td>
<td>0.125</td>
<td>≤0.008-1</td>
<td>0.008-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
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<td>0.047</td>
<td>0.012-0.06</td>
<td>0.002-32</td>
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</tr>
<tr>
<td></td>
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<td>&gt;8</td>
<td>0.5-&gt;8</td>
<td>0.25-8</td>
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<tr>
<td></td>
<td>Erythromycin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.5-&gt;256</td>
<td>0.016-256</td>
<td></td>
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<tr>
<td></td>
<td>Azithromycin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.19-&gt;256</td>
<td>0.016-256</td>
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<td>Clarithromycin</td>
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<td>&gt;256</td>
<td>0.25-&gt;256</td>
<td>0.016-256</td>
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<td><strong>Erythromycin-susceptible GBS (n=10)</strong></td>
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<td>Solithromycin</td>
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<tr>
<td></td>
<td>Penicillin</td>
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<td>Erythromycin&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.047</td>
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<td>Clarithromycin</td>
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<td>0.016-256</td>
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<sup>a</sup> Broth microdilution test  
<sup>b</sup> Etest

Table 2. MIC<sub>50</sub> and MIC<sub>90</sub> of solithromycin and comparator drugs against *S. agalactiae* strains with defined macrolide-resistant genotypes

<table>
<thead>
<tr>
<th>Drug</th>
<th>ermB (26)</th>
<th>mefA/E (22)</th>
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<tr>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
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<tr>
<td>Solithromycin</td>
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<td>0.06</td>
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<td>Penicillin</td>
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<td>Azithromycin</td>
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<td>Clarithromycin</td>
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<sup>a</sup> Broth microdilution test  
<sup>b</sup> Etest
Table 3. Distribution of the alp genes among the observed GBS serotypes

<table>
<thead>
<tr>
<th>Surface alpha-like protein genes (no. of isolates)</th>
<th>No. of isolates per serotypes</th>
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<tr>
<td></td>
<td>Ia</td>
</tr>
<tr>
<td>alp2-3 (30)</td>
<td>2</td>
</tr>
<tr>
<td>rib (33)</td>
<td>4</td>
</tr>
<tr>
<td>epsilon (22)</td>
<td>6 *</td>
</tr>
<tr>
<td>alpha c (10)</td>
<td>2</td>
</tr>
<tr>
<td>alp 4 (14)</td>
<td>1</td>
</tr>
</tbody>
</table>

*p<0.05;**p<0.01