Determination of in vitro activity of solithromycin at different pHs and its intracellular activity tested against clinical isolates of Neisseria gonorrhoeae from a laboratory collection.

Julia Mallegol¹, Prabhavathi Fernandes², Christine Seah¹, Cyril Guyard¹,³,⁴ and Roberto G. Melano¹,³,⁴*

¹Public Health Ontario Laboratory, Toronto; ²Cempra Inc., Chapel Hill, North Carolina, USA; ³Department of Laboratory Medicine & Pathobiology, University of Toronto, Toronto; ⁴Mount Sinai Hospital, Toronto, Ontario, Canada.

Running title. Antigonococcal activity of solithromycin

Keywords. solithromycin; Neisseria gonorrhoeae; intracellular activity; azithromycin-resistance; pH.

*Corresponding Author:

Roberto G. Melano
Public Health Ontario - Toronto
81 Resources Road, Rm. 244
Toronto, ON M9P 3T1
Phone: 416-235-6136
Fax: 416-235-6281
Email: roberto.melano@oahpp.ca
Abstract

We evaluated the activity of solithromycin against 196 clinical gonococcal isolates collected at the Public Health Ontario Laboratories, Toronto, Canada, including isolates with different levels of azithromycin resistance, as well as the role of pH in MIC determinations using pH-adjusted agar plates (pH range, 5.6 to 7.6). In vitro invasion assays were performed using monolayers of HeLa epithelial cells and clinical gonococci displaying different azithromycin MICs; infected cultures were treated with solithromycin and its intracellular activity was determined by colony forming unit assays after 3 and 20 hours of exposure. Solithromycin displayed MIC<sub>50</sub> and MIC<sub>90</sub> of 0.0625 and 0.125 µg/ml, respectively, making its activity at least 4-fold higher than azithromycin. Clinical isolates with elevated MICs for azithromycin (MICs of ≥2,048 µg/ml, and 4-8 µg/ml) showed solithromycin MIC values of 8 and 0.5 µg/ml, respectively. In contrast to azithromycin, solithromycin MICs were not significantly affected by acidic pHs, suggesting more stability at lower pH. Moreover, when intracellular N. gonorrhoeae were incubated with solithromycin at 4X, 1X and 1/4X the MIC, the exposure to solithromycin resulted in the progressive loss of viability of most isolates over time. The intracellular activity of solithromycin combined with the low MICs to this agent indicates it could be an attractive option for gonorrhoea treatment if clinical trials in development reveal that this drug can be used safely in adult indications, especially when multidrug resistant clinical isolates are now emerging.
Introduction

Among the few newer antimicrobials developed in recent years, solithromycin (CEM-101), a potent novel fluoroketolide currently in clinical development, has a reported high potency against Gram-positive pathogens, including macrolide-resistant isolates (1-3) and various fastidious Gram-negative (4-6). *In vitro* studies have shown that solithromycin has activity comparable or superior to telithromycin, erythromycin, azithromycin, and clarithromycin against different bacterial species (4, 7). Moreover, Golparian et al. have reported that the *in vitro* activity of solithromycin is superior to azithromycin and many other antimicrobials against international reference strains and clinical gonococcal isolates including some with high-level antimicrobial resistance (5). In this study we investigated the *in vitro* activity of solithromycin at different pHs against a gonococcal collection, including azithromycin-susceptible and -resistant isolates. Using the carcinoembryonic antigen cell adhesion molecule (CEACAM)-mediated uptake of colony opacity-associated adhesins (Opa)-expressing *N. gonorrhoeae* as a well characterized model of bacterial invasion (8), we analysed the intracellular activity of solithromycin on gonococci invasion into cervical epithelial cells. This study was initiated to provide comprehensive data on the activity of solithromycin and facilitate its development as a potential alternative chemotherapeutic agent in the eradication of *N. gonorrhoeae*.

Material and methods

Antimicrobial susceptibility testing
Public Health Ontario (PHO) Laboratories provide primary testing for dedicated sexually transmitted infections clinics throughout the province of Ontario, Canada. This includes both culture and nucleic acid amplification testing (NAAT) for *N. gonorrhoeae*. PHO also performs susceptibility testing for all *N. gonorrhoeae* isolates identified at other hospital and private laboratories across the province. In that context, a total of 196 *N. gonorrhoeae* were selected from the clinical isolates collected from 2008 to 2011. Among those isolates, 67 were reported in our previous susceptibility profiles study (8 antibiotics, including penicillin, tetracycline, ciprofloxacin, erythromycin, azithromycin, spectinomycin, ceftriaxone and cefixime) (9). Isolates susceptible to, with reduced susceptibility to, and resistant to azithromycin were also included. Primary specimens and isolates received for confirmation of *N. gonorrhoeae* were subcultured on New York City (NYC) agar (10) and incubated for 24 to 72 h in 5% CO₂ at 35 to 37°C. Gram stain, oxidase, and carbohydrate utilization tests (glucose, maltose, sucrose, and O-nitrophenyl-β-d-galactopyranoside [ONPG]) were performed. Identification of *N. gonorrhoeae* also included testing growth on blood agar at 22°C and on nutrient agar at 35.5°C. All isolates were cultured on GC agar and 1% defined growth supplement at 37°C in 5% CO₂ for 20 to 24 h and stored at -86°C. Each sample was subcultured twice on NYC agar before antimicrobial testing. The MICs of solithromycin and azithromycin were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (11). Since CLSI does not include breakpoints for azithromycin, we used the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for interpretation of the results (S, ≤0.25 µg/ml; R, ≥1 µg/ml) (available at...
http://www.eucast.org/clinical_breakpoints/, accessed on January 6th, 2013). *N. gonorrhoeae* strains WHO L (intermediate resistance to azithromycin, 0.5 µg/ml) and P (resistant to azithromycin, 2 µg/ml) were used as quality control strains (12). For susceptibility testing of selected *N. gonorrhoeae* isolates against solithromycin and azithromycin at different pHs, GC agar with pHs ranging from 5.6 to 7.6 were in house prepared and buffered using 0.1 M potassium phosphate buffers (5.6 to 7.6). The final pH values of the GC agar plates were confirmed using a flat pH surface electrode.

Molecular characterization of macrolide resistance

The presence of acquired 23S rRNA methylases (*ermA*, *ermB*, *ermC* and *ermF* genes), efflux pump (*mefA/E* gene), macrolide 2′-phosphotransferase (*mphA* gene), erythromycin esterases (*ereA* and *ereB* genes) and possible mutations in the chromosomal *mtrR* gene/promoter and riboproteins L4 and L22 (*rplD* and *rplV* genes) were tested as described (9) in selected isolates used for intracellular activity assays. Mutations in the 4 copies of 23S rRNA *rrl* genes identified in *N. gonorrhoeae* were also analyzed by DNA sequencing of PCR amplicons. Briefly, for each 23S rRNA copy, common primer inside each copy of the *rrl* genes and specific external primers on the closer open reading frame downstream to each *rrl* genes were designed from the complete *N. gonorrhoeae* NCCP11945 genome (accession number NC_011035) (Table 1). Using the first PCR amplicons as DNA template, nested PCRs were next performed using a set of common primers (NG23s1905-F and NG23s2769-R, Table 1) internal to 23S rRNA gene (including the peptidyl-transferase loop of the domain V). The second-round PCR
amplicons were sequenced to identify point mutations associated with macrolide resistance. The same approach was used for analysis of domain II of 23S rRNA gene, particularly the position A752 (NG752-R and NG752-F, Table 1) potentially involved in solithromycin binding.

Intracellular activity of solithromycin

Five *N. gonorrhoeae* clinical isolates demonstrating susceptibility (GN48) and resistance (GN640, GN641, GN642 and GN726) to azithromycin were used for the experiments and grown as described above. For infection assays, stably transfected HeLa line cells (human endocervical epithelial) expressing human CEACAM1 receptor (8, 13, 14) were grown to 70–80% confluence in 24-well plates (Costar 24 well flat bottom plate, Corning Incorporated, Lowell, MA) containing 1 ml/well of RPMI 1640 medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat inactivated fetal calf serum and 1% glutamine (Life Technologies), and incubated at 37°C in presence of 5% CO₂ for 48 h. Cells were washed three times in serum-free RPMI-1640 prior to infection. Gonococci were scraped from fresh overnight culture plates, resuspended in 1 ml of phosphate-buffered saline (PBS) containing 1mM MgCl₂ and 0.5 mM CaCl₂ (pH 7.0), washed once by pelleting and resuspended to a concentration of 3.2 x 10⁷ cells/ml. Gonococci were added to the cells at a multiplicity of infection (MOI) of 80:1 in a volume of 1 ml per well. Samples were subjected to centrifugation for 5 min at 500 rpm to promote bacterial association with HeLa cells. Then the cultures were incubated for 1 h at 37°C in 5% CO₂. After 3 washes in serum-free RPMI to remove non-
adherent bacteria, gentamicin was added (50 µg/ml final concentration) for 1 h to eradicate extracellular bacteria. To confirm that the gentamicin treatment was sufficient to kill extracellular gonococci in our infection assays, the MICs for gentamicin were initially determined on bacterial suspensions of the five clinical isolates studied. MIC values ranged between 3 to 8 µg/ml, suggesting that all of the isolates should be killed by the 50 µg/ml concentration of gentamicin (which is at least 8 times higher than MIC determined) used in the infection assay. Cells were washed three times in serum-free RPMI-1640 to remove extracellular bacteria and gentamicin, and were incubated with various concentrations of solithromycin (4X, 1X, 1/4X the MIC) or media/buffer alone at 37°C in 5% CO₂. After 0, 3 and 20 h incubation, cells were washed 3 times with PBS containing 1mM MgCl₂ and 0.5 mM CaCl₂ (pH 7.0), and 1% saponin was then added to permeabilize and lyse HeLa cells for 15 min at 37°C in 5% CO₂ before dilution and plating onto GC agar. Plates were incubated overnight to quantify viable intracellular bacteria (13). All assays were performed in quadruplicate with two independent biological duplicates.

Results

Antigonoococcal activity of solithromycin

Overall, solithromycin demonstrated superior potency against N. gonorrhoeae when compared to azithromycin, with lower MICs than azithromycin against the vast majority of isolates evaluated (Figure 1). Most of the isolates (96%) showed MICs for solithromycin ranging from ≤0.015 µg/ml to 0.125 µg/ml whereas 75% of the isolates...
showed MICs for azithromycin ranging from 0.125 µg/ml to 0.5 µg/ml. None of the tested isolates had solithromycin MICs greater than 8 µg/ml. *N. gonorrhoeae* MICs for solithromycin ranged from ≤0.015 to 8 µg/ml compared to the MICs of azithromycin which ranged from ≤0.031 to ≥2,048 µg/ml. Solithromycin had a MIC$_{50}$ of 0.0625 µg/ml and a MIC$_{90}$ of 0.125 µg/ml, making its activity 4-fold higher than azithromycin (MIC$_{50}$ of 0.25 µg/ml and a MIC$_{90}$ of 0.5 µg/ml). Clinical isolates with elevated MICs for azithromycin (MICs of ≥2,048 and 4-8 µg/ml) showed solithromycin MICs of 8 µg/ml (≥256-fold difference) and 0.25-0.5 µg/ml (16-fold difference), respectively (Figure 1).

**Role of pH in the activity of solithromycin and azithromycin**

We investigated the effect of pH on the activity of solithromycin and azithromycin against *N. gonorrhoeae* using pH-adjusted agar plates (pH range, 5.6 to 7.6). This range was selected to cover the values at which the antibiotics could be exposed in the extracellular milieu or intracellularly for *N. gonorrhoeae*. Unlike erythromycin, the 15-member azalide ring of azithromycin is acid stable (15). For the short duration of the assay azithromycin and solithromycin are both stable at the pHs tested, but azithromycin is less stable at an acid pH for longer periods of time as the cladinose sugar could be lost (Cempra’s unpublished data). We compared the susceptibilities of six *N. gonorrhoeae* isolates which displayed the highest solithromycin and azithromycin MIC values at different pHs. Control strains and clinical isolates were not able to grow on plates with pHs 5.6 and 6.0 in the absence of antibiotics. Therefore, data are only presented for plates prepared at pH 6.4, 6.8, 7.2, and 7.6. All isolates with intermediate susceptibility (0.5...
µg/ml) or resistant (≥1 µg/ml) to azithromycin displayed 2- to 16-fold higher MICs at lower pHs, compared to pH 7.2 (control plate). In contrast, solithromycin MICs remained low throughout the pH range evaluated and were not significantly affected by acidic pHs (Figure 2).

**Macrolide resistance mechanisms**

Three different mechanisms involved in macrolide resistance have been described in *N. gonorrhoeae*: efflux systems (16, 17), modification of the ribosomal target by methylases (18) and ribosomal modification by point mutations in the macrolides targets (19). The presence of these mechanisms was investigated on five representative *N. gonorrhoeae* clinical isolates displaying susceptibility (GN48) and various resistance levels (GN640, GN641, GN642 and GN726) to azithromycin (Table 2). High level macrolide resistance (strain GN640) was associated to target mutation (A2143G in all 4 copies of 23S rRNA rrl gene) (20, 21). Low level resistance was linked to a different mutation in the target (C2599T) but also in the *mtrR* gene and riboprotein L22 (Table 2). No mutations in domain II of 23S rRNA gene, position A752, were detected in any isolate. Isolates GN726 and GN48 were wild type for all the genes tested. Macrolide resistance mediated by acquired efflux pump, methylases, and esterases was not detected in neither isolate. Mutations affecting MtrR (repressor of the chromosome-encoded MtrCDE efflux pump) was only found in strain GN640.

**Intracellular activity of solithromycin against gonococci**
The intracellular activity of solithromycin against these five *N. gonorrhoeae* isolates was next tested. Following incubation within HeLa cells expressing CEACAM1 receptor, internalized *N. gonorrhoeae* isolates were exposed to solithromycin at 4X, 1X and 1/4X of their respective MIC. To rule out a possible growth-suppressive effect of solithromycin on epithelial cells in our experimental conditions, we first verified if increasing concentrations of solithromycin could affect the viability of epithelial cells. Solithromycin concentrations ranging from 0.0625 to 32 µg/ml were shown to not affect the viability of HeLa cells when evaluated by trypan blue staining (data not shown). As presented in Figure 3, all isolates were internalized with similar efficiency at time zero. Exposure to solithromycin at 4X and 1X the MIC resulted in the progressive loss of viability of all isolates at 20 h compared to time zero with the exception of GN726, for which viability remained stable at 20 h compared to 3 h (Figure 3D). The loss of viability for all other isolates ranged from 99% to 100% of colony forming units (CFU) per millilitre. Interestingly, in the untreated culture controls, GN726 replicated by 7 log units at 20 h while GN48, GN642, GN640, GN641 ranged from 3 to 5 log units.

Concentrations of solithromycin equal to 1/4X the MIC for each isolate were uniformly ineffective in killing intracellular organisms. However, 3 of *N. gonorrhoeae* clinical isolates (GN641, GN642, and GN726) displayed a significantly lower viability at 1/4X the solithromycin MIC compared to no antibiotic at 20 h (P-value < 0.05).

**Discussion**
Since the early 1990s, clinical isolates with decreased susceptibility to azithromycin have been observed in several countries (22, 23). Interestingly, the Gonococcal Isolate Surveillance Project data for 2007 showed that the distribution of azithromycin MICs shifted toward the higher MICs in the United States (24). However, the use of a single dose of 1 g azithromycin in combination therapy (with cephalosporins) is still the recommended. The emergence of multidrug resistant and untreatable *N. gonorrhoeae* highlights the importance of identifying alternatives using known antibiotics or new antimicrobials for effective treatment of gonorrhoea infections. Among known antibiotics, gentamicin and ertapenem have been recently evaluated as potential drugs for the treatment of gonorrhoea (25-27).

Recently, solithromycin, a new fluoroketolide with high affinity for bacterial ribosomes, has been evaluated as a potential new antimicrobial for gonorrhoea treatment (5). In the present study, we measured for the first time the potency of solithromycin against a variety of Canadian clinical isolates recently collected in Ontario and displaying a broad range of susceptibility to azithromycin, including high level of resistance (e.g. MIC ≥2,048 µg/ml). We found that solithromycin has higher intrinsic antigonococcal activity (lower MICs) than azithromycin. It is known that the C5 desosamine sugar residues in macrolides and ketolides interact with positions A2058-A2059 (*E. coli* nucleotide numbering; A2142-A2143 in *N. gonorrhoeae* numbering) of the bacterial 23S rRNA (28). This explains why mutations in these nucleotides can cause resistance to these antibiotics and it is consistent with the high azithromycin MIC observed with the *N. gonorrhoeae* GN640 isolate (A2143G) of our study. In the case of ketolides, the alkyl-
aryl side chain attached at the C-11 and C-12 carbon atoms also interacts with the A752-U2609 base pair (E. coli nucleotide numbering; A750-U2597 in N. gonorrhoeae numbering) and, in the particular case of solithromycin, the fluorine atom positioned near the glycosidic bond (atom N-1) of C2611 (E. coli numbering; C2599 in N. gonorrhoeae numbering) can potentially contribute to the drug binding (29). Compared to azithromycin, these multiple interactions may explain the better anchoring of solithromycin to the ribosome, even in presence of mutations in key positions (i.e. A2142-A2143). Because of that, lower MICs are expected as observed in isolate GN640 (MIC ≥2048 µg/ml for AZM vs. 8 µg/ml for solithromycin). Mutation C2599T may affect the interaction of the solithromycin fluorine atom with the ribosome, reducing the susceptibility to solithromycin (from ≤0.015 µg/ml in the wild type GN48 to 0.25 µg/ml in GN641). No mutations affecting the binding of the solithromycin alkyl-aryl side chain were detected by sequencing.

Moreover, with a subset of selected isolates we also investigated the pH stability and the intracellular activity of solithromycin using a tissue culture model of cervical cell lines. Our results also showed stable activity of solithromycin at different pH values against N. gonorrhoeae, whereas azithromycin showed a marked decrease in potency against all isolates tested when the pH was decreased from 7.6 to 6.4 (Figure 2). Several studies have previously demonstrated that pH affect azithromycin activity in vitro (30-32). In contrast, our results showed stable anti-gonococcal activity of solithromycin between pH 6.4 and 7.4, suggesting solithromycin would be likely to have potent activity in acidic compartments such as endosomes/lysosomes compared to azithromycin. In agreement
with our results, a previous study has demonstrated that acidic pH has a reduced inhibitory effect on solithromycin activity compared to azithromycin activity (30). This same study also showed that solithromycin has high intracellular activity against 
*Staphylococcus aureus, Listeria monocytogenes* and *Legionella pneumophila* due to high intracellular accumulation. In intracellular assays, solithromycin demonstrated efficient activity against all tested isolates with the exception of GN726 (Figure 3). When exposed to 4X MIC, this specific clinical isolate did not show a significant decrease of viability at 20 h compared to 3 h. This phenotype is not explained by an increase in resistance to macrolides since GN726 does not present any mutations in the drug target, the 4 copies of the 23S rRNA gene, nor it does express plasmid-mediated efflux pumps or produces methylases associated with macrolide resistance. The reduced intracellular activity of solithromycin against this specific isolate may be explained by an elevated intracellular fitness as it replicated more efficiently by 2-3 log units within epithelial cells than others isolates in the untreated culture controls at 20 h. To rule out viable but not internalized gonococci affecting the intracellular assay results we used gentamicin treatment, which is a standard method to quantify viable intracellular *N. gonorrhoeae* (13, 33). Considering the gonococcal MICs, the used concentration (50 µg/ml) was sufficient to kill all not internalized isolates, consistent with a previous study showing that, upon gentamicin treatment, nonviable *N. gonorrhoeae* fail to recruit F-actin and were not internalized by hosts cells (34). In conclusion, solithromycin was demonstrated to be stable and potent against *N. gonorrhoeae*, even against isolates with high azithromycin MICs. In the phase 2 of a
clinical trial, solithromycin has been found to be 100% effective against *N. gonorrhoeae* in all culture tested (urethra, oropharynx and rectal) using 1.2 g and 1 g as a single dose (Cempra’s unpublished data). Our *in vitro* results and these phase 2 studies show the potential value of solithromycin for use in the treatment of gonorrhoea, especially when multidrug resistant clinical isolates displaying full resistance to azithromycin and ESC are now emerging.

**Acknowledgements**

Part of this study was presented at the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco (poster E781), and at the IDWeek, San Diego, CA, USA, 2012 (poster E-1614).

We thank Vanessa Allen (Public Health Ontario) for providing the gonococcal isolates used in this study, and Scott D Gray-Owen (Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada) for the stably transfected HeLa epithelial cell used in this study and for expert technical assistance.

**Funding**

This work was supported by Cempra Inc.

**Transparency declarations**

P.F. is the CEO of Cempra Inc. All other authors: none to declare.
TABLE 1. Oligonucleotides used for amplification and sequencing of domains II and V of 23S rRNA gene.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>23S rRNA - Domain V</td>
<td>External specific primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NG23S1-F</td>
<td>GGCTATGAAGGCGGCGATT</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>NG23S2-F</td>
<td>TTTCAAGTGAGTAATGTACACC</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>NG23S3-F</td>
<td>CAATCCGCAATCTGCCGA</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>NG23S4-F</td>
<td>CTCTCCGATCCGAACTCG</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Internal common primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NG23S-R</td>
<td>GAAGATGTGCGAAGCATCGGA</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Nested PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NG23s1905-F</td>
<td>ACGGTCTAAGGACGCA</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>NG23s2769-R</td>
<td>TTCATCTTCAGGCGAGTT</td>
<td>9</td>
</tr>
<tr>
<td>23S rRNA - Domain II</td>
<td>Internal common primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NG752-R</td>
<td>CAACGACTTACATTCAGTAGC</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Semi-nested PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NG752-F</td>
<td>TTCTGATACCTCCAGCACAC</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a* External specific primers were used for specific amplification of each copy of 23S rRNA gene (4 copies in *N. gonorrhoeae*) together with each internal common primer (NG23S-R for amplification of domain V, and NG752-R for domain II). The obtained amplicons were used for the subsequent nested or semi-nested assay.

*b* For the semi-nested PCR, primers NG752-F and -R were used.
TABLE 2. Characterization of macrolide resistance mechanisms on five *N. gonorrhoeae* clinical isolates.

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC (µg/ml)</th>
<th>Mutation</th>
<th>Gene affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN48</td>
<td>0.062 (S)</td>
<td>ND</td>
<td>None</td>
</tr>
<tr>
<td>GN640</td>
<td>≥2048 (R)</td>
<td>≥256</td>
<td>A2143G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G45D</td>
</tr>
<tr>
<td>GN641</td>
<td>3 (R)</td>
<td>64</td>
<td>C2599T</td>
</tr>
<tr>
<td>GN642</td>
<td>1 (R)</td>
<td>3</td>
<td>A89D</td>
</tr>
<tr>
<td>GN726</td>
<td>1 (R)</td>
<td>3</td>
<td>None</td>
</tr>
</tbody>
</table>

AZM, azithromycin; ERY, erythromycin; SOLI, solithromycin; ND, not determined.

*a* S, susceptible; R, resistant, according to the EUCAST breakpoints: S, ≤0.25 µg/ml; I, 0.5 µg/ml; R, ≥1 µg/ml.

*b* All the chromosomal targets studied were wild-type.

*c* *N. gonorrhoeae* numbering. Positions A2143 and C2599 correspond to A2059 and C2611 in *Escherichia coli* numbering, respectively.

*d* The four rrl alleles contained the same mutation.
**FIGURE CAPTIONS**

**Figure 1.** MIC (µg/ml) distribution of solithromycin and azithromycin for a collection of 196 clinical *N. gonorrhoeae* isolates. * Lowest dilution tested for azithromycin.

**Figure 2.** Susceptibilities to solithromycin and azithromycin of *N. gonorrhoeae* isolates grown on pH-adjusted GC agar plates. AZM, azithromycin. MIC determinations were performed in pH-adjusted GC plates. EUCAST breakpoints for azithromycin were used: S, ≤0.25 µg/ml; I, 0.5 µg/ml; R, ≥1 µg/ml.

**Figure 3.** Intracellular activity of solithromycin (SOLI) against five *N. gonorrhoeae* clinical isolates. *N. gonorrhoeae* isolates internalised within HeLa cell line were exposed to various concentrations of solithromycin. At indicated time points, samples were harvested and bacteria were enumerated by CFU assays. 4X, exposure to 4 times solithromycin MIC; 1X, exposure to 1 time the MIC; 1/4X, exposure to 1/4 times the MIC. * Student’s t-test P value < 0.05 no solithromycin vs. 1/4X solithromycin.
REFERENCES


Figure 1. MIC (μg/ml) distribution of solithromycin and azithromycin for a collection of 196 clinical *N. gonorrhoeae* isolates. * Lowest dilution tested for azithromycin.
Figure 2. Susceptibilities to A. azithromycin and B. solithromycin of *N. gonorrhoeae* isolates grown on pH-adjusted GC agar plates. AZM, azithromycin. MIC determinations were performed in pH-adjusted GC plates. EUCAST breakpoints for azithromycin were used: S, ≤0.25 μg/ml; I, 0.5 μg/ml; R, ≥1 μg/ml.
Figure 3. Intracellular activity of solithromycin (SOLI) against five *N. gonorrhoeae* clinical isolates. *N. gonorrhoeae* strains internalised within HeLa cell line were exposed to various concentrations of solithromycin. At indicated time points, samples were harvested and bacteria were enumerated by CFU assays. 4X, exposure to 4 times solithromycin MIC; 1X, exposure to 1 time the MIC; 1/4X, exposure to 1/4 times the MIC. * Student’s t-test P value < 0.05 no solithromycin vs 1/4X solithromycin.