

## Azithromycin iv pharmacodynamic parameters predicting *Streptococcus pneumoniae* killing in epithelial lining fluid versus serum: an *in vitro* pharmacodynamic simulation

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**Objectives:** To investigate the azithromycin pharmacodynamic parameters predicting bacterial killing in epithelial lining fluid (ELF) versus serum against macrolide-susceptible and -resistant *Streptococcus pneumoniae* isolates (with different resistance genotypes), through the simulation of concentrations achieved after a 500 mg intravenous (iv) once a day regimen.

**Methods:** An *in vitro* computer-controlled pharmacodynamic simulation of human azithromycin concentrations in serum and ELF was carried out, and colony counts were determined over 24 h. Four strains with MIC values (mg/L) of 0.5 [*mef(A)* and *erm(B)* negative], 2 [*mef(A)* positive and *erm(B)* negative], 8 [*mef(A)* positive and *erm(B)* negative] and 256 [*mef(A)* negative and *erm(B)* positive] were used.

**Results:** Significant ( $P < 0.05$ ) azithromycin antibacterial activity versus antibiotic-free controls was found in serum and ELF against the susceptible and *mef(A)* positive strains, but not against the *erm(B)* positive strain.  $AUC_{0-24}/MIC$  values around or higher than 25 were needed to achieve (time to 99.9% reduction of initial inocula of around 6 h) and maintain (24 h inocula reduction  $\geq 3 \log_{10} \text{cfu/mL}$ ) bactericidal activity without regrowth. This was achieved only with the susceptible strain in serum, but also with the *mef(A)* positive strain exhibiting an MIC of 2 mg/L in ELF.

**Conclusions:** The results of this study support that the suggested breakpoint for susceptibility ( $\leq 2 \text{ mg/L}$ ) may be adequate to predict *S. pneumoniae* eradication with ELF but not with serum concentrations obtained after a 500 mg iv once a day regimen.

Keywords: *in vitro* models, antipneumococcal bactericidal activity, resistance genotypes

### Introduction

Because of its basic structure, azithromycin (but not other macrolides) is actively taken up by white blood cells<sup>1</sup> that chemotactically carry the azalide to the site of infection. After contact with encapsulated microorganisms that are difficult to phagocytose, such as *Streptococcus pneumoniae*, the drug accumulated in the lysosome migrates to the edge of the cell and is released by degranulation.<sup>2</sup> This extensive uptake and slow release of azithromycin from tissues,<sup>3</sup> and a very large volume of distribution, are responsible for its long half-life [ranging from 14.9 to 76.8 h after a single 500 mg intravenous (iv) dose or at steady-state].<sup>4</sup> These data together with protein binding (50% at concentrations from 0.02 to 0.05 mg/L, but only 7% at concentrations  $\geq 1 \text{ mg/L}$ )<sup>5</sup> indicate that high levels

of the free-drug are available at infectious sites after a 500 mg iv regimen, achieving serum  $C_{\text{max}}$  values of  $\sim 4.0 \text{ mg/L}$ ,<sup>4</sup> serum  $AUC_{0-24}$  ranging from 9.6 to 17.7 mg·h/L,<sup>4,6</sup> and epithelial lining fluid (ELF)  $C_{\text{max}}$  and  $AUC_{0-24}$  values of 2.86 and 45.8 mg·h/L, respectively.<sup>7</sup>

It has been suggested that, in respiratory tract infections, concentrations at the site of infection, such as ELF, are more predictive of antimicrobial effects of azithromycin against extracellular pathogens such as *S. pneumoniae*<sup>8</sup> and that an *in vivo*  $AUC/MIC$  ratio of at least 10 is needed for an *in vivo* bacteriological response against *S. pneumoniae* in the immunocompetent host.<sup>7</sup> A previous report on serum and ELF simulations of free-drug concentrations (using a single protein binding value of 50%) after azithromycin oral regimens showed absence of killing at 24 h against azithromycin-resistant *S. pneumoniae* ( $MIC \geq 2 \text{ mg/L}$ ;

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**Table 1.** Characteristics of strains used in the study

Strain no.	Serotype	MIC (mg/L)	Resistance genotype	
			<i>mef(A)</i>	<i>erm(B)</i>
1	14	0.5	–	–
2	23	2	+	–
3	23	8	+	–
4	non-typeable	256	–	+

serum or ELF  $AUC_{0-24}/MIC < 5$ ), regardless of the resistance genotype [*mef(A)* or *erm(B)*].<sup>9</sup>

In the present study we have explored the azithromycin pharmacodynamic parameters predicting bacterial killing in ELF versus serum against macrolide-susceptible and -resistant *S. pneumoniae* (with different resistance genotypes), through the simulation of concentrations achieved after a 500 mg iv regimen.

### Material and methods

#### Strains

Four *S. pneumoniae* clinical isolates from the Spanish Pneumococcal Infection Network (G03/103) were used. MICs were determined five times following CLSI (formerly NCCLS) recommendations<sup>10</sup> and modal values were considered. *mef(A)* and *erm(B)* genotypes were determined using PCR as described previously.<sup>11</sup> Table 1 shows serotype, susceptibility and resistance genotype of the four strains used in the study.

#### Antibiotic

Azithromycin laboratory reference standard was supplied by Pfizer (Groton, CT, USA).

#### In vitro kinetic model

A previously described dynamic model was used in the study.<sup>12</sup> Briefly, the model consisted of two compartments, with hollow fibre capsule filters (FiberFlo; Minntech Corp., Minneapolis, MN, USA) as the second compartment (which included the peripheral compartment as the infection site). The model was designed to expose bacteria to changing antibiotic concentrations, without dilution of the bacterial inoculum together with the antibiotic. A computer-controlled syringe pump (402 Dilutor Dispenser; Gilson S.A, Villiers-le-Bel, France) was used to simulate the 1 h infusion of azithromycin iv until the corresponding serum or ELF  $C_{max}$  was achieved in the central compartment ( $C_c$ ). Peristaltic pumps (Masterflex; Cole-Parmer Instrument Co., Chicago, IL, USA) were used to continually replace the antibiotic-containing medium with fresh medium, and to circulate the medium between and within the two compartments. Both compartments were maintained at 37°C for the duration of the experiment.

Flow rates in the peristaltic pumps were synchronized all over the simulated period using the Win Lin software (Cole-Parmer Instrument Co.).

After 4 h, to mimic the concentrations achieved in human ELF from 4 to 24 h<sup>7</sup> an additional reservoir, subcompartment 0 ( $S_0$ ), was used. The volume of  $S_0$  was calculated according to the volume of  $C_c$  (600 mL) and the flow rate for azithromycin.<sup>8</sup>

Todd–Hewitt broth (Difco laboratories, Detroit, MI, USA) supplemented with 0.5% yeast extract (Difco laboratories) was used as the medium throughout the pharmacodynamic simulation.

#### Pharmacokinetic simulations

Steady-state pharmacokinetic profiles in serum and ELF after an azithromycin 500 mg iv regimen were simulated over 24 h.<sup>7,13</sup> Target  $C_{max}$  in serum and ELF were 3.63 and 2.86 mg/L, respectively.<sup>7,13</sup> Target  $AUC_{0-24}$  in serum was 13.6 mg·h/L, representing the mean of published data (9.6–17.7 mg·h/L).<sup>4,6</sup> Target  $AUC_{0-24}$  in ELF was 45.8 mg·h/L.<sup>7</sup> The target serum concentrations were obtained from azithromycin iv serum concentration-versus-time data.<sup>13</sup> The profile was divided into three quasi-linear portions with apparent half-lives of 0.39 h ( $K_e$ ; 1.8 h<sup>-1</sup>) from 1 to 2 h, of 1.73 h ( $K_e$ ; 0.4 h<sup>-1</sup>) from 2 to 3 h (corresponding to the initial rapid decline in concentration and redistribution into the tissues of azithromycin) and of 10.5 h ( $K_e$ ; 0.06 h<sup>-1</sup>) from 3 to 24 h (representing further distribution and elimination). The clearance used in the ELF profile was calculated using a half-life of 68 h ( $K_e$ ; 0.01 h<sup>-1</sup>).

#### Pharmacokinetic analysis

Pharmacokinetic analysis was performed in triplicate, in bacteria-free simulations under the same experimental conditions. Samples (0.5 mL) from the peripheral compartment were obtained at 0, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 24 h. Concentrations were determined in triplicate by bioassay using *Micrococcus luteus* ATCC 9341 as the indicator organism.<sup>14</sup> Standards were prepared in Todd–Hewitt broth supplemented with 0.5% yeast extract. The lower limit of detection was 0.06 mg/L. The correlation coefficient was >0.99 and intra-day and inter-day coefficients of variation were 9.8% and 12.5%, respectively, at a level of 0.75 mg/L.

Azithromycin concentrations were analysed by a non-compartmental approach using WinNonlin Professional program (Pharsight, Mountain View, CA, USA).  $C_{max}$  was obtained directly from the observed data. The area under the concentration–time curve from 0 to 24 h ( $AUC_{0-24}$ ) was calculated by the trapezoidal rule.

#### Experiments

Prior to each experiment, 1–2 colonies from a fresh passage on Mueller–Hinton agar supplemented with cations and 5% lysed sheep blood were inoculated in 50 mL of Todd–Hewitt broth supplemented with 0.5% yeast extract. The suspension was allowed to grow to a density of 10<sup>7</sup> cfu/mL, as measured using a UV-spectrophotometer (Hitachi U-1100). Of this inoculum, 30 mL was introduced into the peripheral compartment of the *in vitro* model 1 h prior to each simulation process to allow the microorganism to adapt to the medium. All initial inocula were in the range 10<sup>7</sup> to 3 × 10<sup>7</sup> cfu/mL.

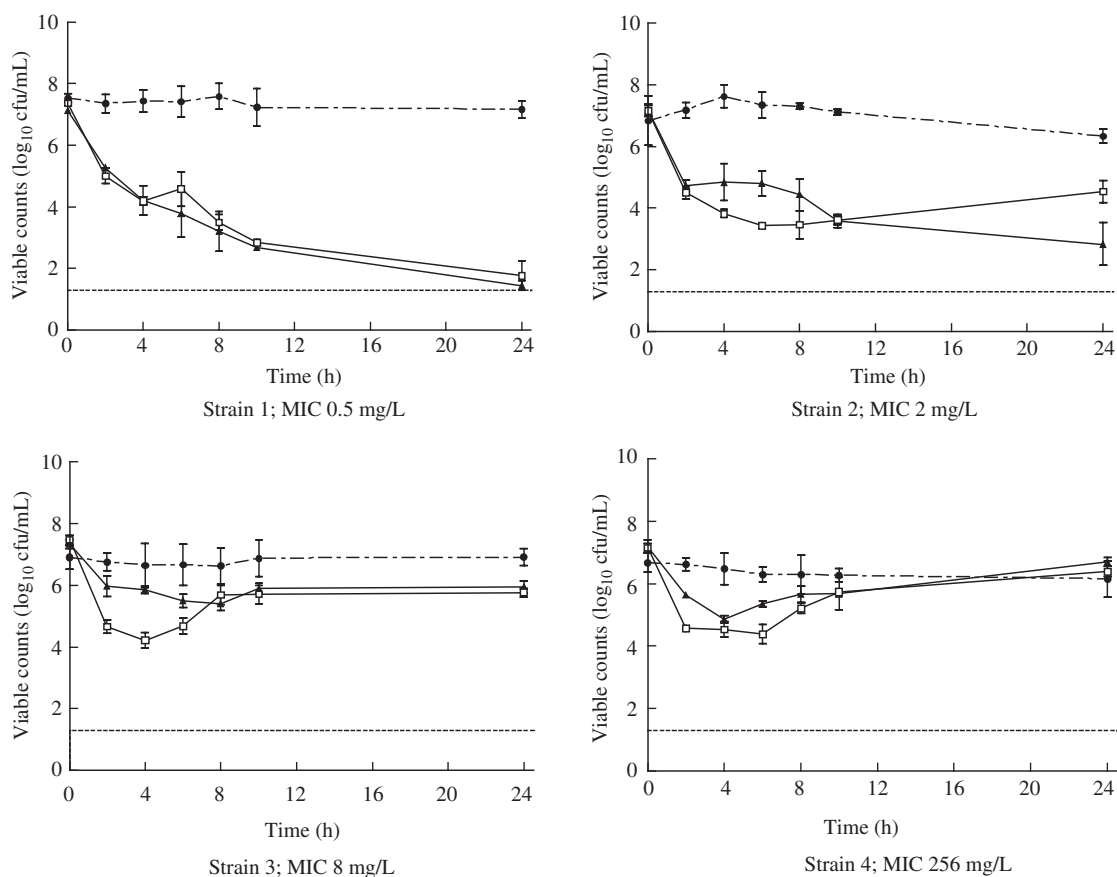
Samples (0.5 mL) from the peripheral compartment were collected at 0, 2, 4, 6, 8, 10 and 24 h. Each sample was serially diluted 10-fold in 0.9% sodium chloride for bacterial counting in supplemented Mueller–Hinton agar with 5% sheep blood which was incubated at 37°C 5% CO<sub>2</sub> for 24 h. The limit of detection was 20 cfu/mL.

Antibiotic-free simulations with each strain were used as controls.

All experiments were performed in triplicate.

#### Statistical analysis

Mean values of cfu/mL were calculated with the three values of colony counts at each time during the 24 h simulation. Reductions in initial inocula at 24 h were calculated by the difference between log<sub>10</sub> cfu/mL initial inocula and log<sub>10</sub> cfu/mL at 24 h. Percentages of reduction of initial inocula at the different sample times were



**Figure 1.** Azithromycin serum (open squares) and ELF (filled triangles) versus antibiotic-free controls (filled circles) antibacterial activity ( $\log_{10}$  cfu/mL over time) against the four *S. pneumoniae* strains. The broken horizontal line represents the limit of detection.

calculated. The time to achieve 99.9% reduction ( $T_{99.9\%}$ ) was determined using linear regression.

Antibacterial activity was also studied using the area under the killing curve (AUKC;  $\text{cfu} \times \text{h/mL}$ ) as a measure of global killing over 24 h. The bacterial growth in antibiotic-free simulations was used as control (AUKC<sub>K</sub>). Differences between AUKC<sub>K</sub> and AUKC obtained in simulations with azithromycin (AUKC<sub>AZ</sub>) were determined. Means  $\pm$  standard deviations were calculated.

Comparisons of 24 h reductions in azithromycin simulations versus controls were analysed using the *t*-test. Inter-strain differences in capability of reduction of control growth curves over 24 h (AUKC<sub>K</sub>–AUKC<sub>AZ</sub>) and 24 h inocula reductions were analysed by the ANOVA. When the ANOVA *P* value was significant, contrast between groups was made by the use of the Tukey–Kramer test to adjust the type I experimental error.

## Results

Experimental  $T_{\max}$  and  $C_{\max}$  (mg/L) in the pharmacodynamic simulations were 1 h and  $3.59 \pm 0.04$  for serum and 24 h and  $2.78 \pm 0.05$  for ELF. AUC<sub>0–24</sub> ( $\text{mg} \cdot \text{h/mL}$ ) was  $13.47 \pm 0.85$  and  $49.09 \pm 0.84$  for serum and ELF, respectively. Serum experimental AUC<sub>0–24</sub> was <1% lower than the target area, and ELF experimental AUC<sub>0–24</sub> was 7% higher than the target area.

Figure 1 and Tables 2 and 3 show the antipneumococcal activity of the serum and ELF azithromycin profiles. In both cases, when exploring antibacterial activity as inocula reduction

at 24 h, azithromycin exhibited significant ( $P < 0.05$ ) activity versus antibiotic-free controls against the susceptible strain or those strains carrying the *mef(A)* gene. No differences ( $P = 0.77$  and  $0.46$  for serum and ELF, respectively) versus antibiotic-free control were found with azithromycin against strain 4, harbouring the *erm(B)* gene. When exploring antibacterial activity through differences between AUKC<sub>K</sub> and AUKC<sub>AZ</sub>, significant ( $P < 0.05$ ) differences were found in serum between all strains. These significant differences were maintained between all strains in the case of ELF, except in the case of strain 3 (MIC = 8 mg/L) versus strain 4 (MIC = 256 mg/L) where statistical differences disappeared.

As shown in Table 2, in serum, no bactericidal activity was obtained against strains 3 and 4 (MIC  $\geq 8$  mg/L), but bactericidal activity ( $T_{99.9\%} = 6.6$  and  $5.5$  h) was obtained against strains 1 and 2. However, in the case of the susceptible strain (strain 1), the bactericidal activity ( $\geq 3 \log_{10}$  reduction) obtained at 6.6 h was maintained and even increased at 24 h ( $5.6 \log_{10}$  reduction), while in the case of strain 2 (exhibiting low resistance; MIC = 2 mg/L) the bactericidal activity achieved at 5.5 h was not maintained and regrowth occurred, the  $\log_{10}$  reduction being 2.6 at 24 h.

In contrast, in ELF, azithromycin bactericidal activity was obtained against strains 1 and 2 at 6.9 and 6.3 h, respectively, and maintained and even increased at 24 h with 5.7 and 4.3  $\log_{10}$  reduction, respectively. Again, no bactericidal activity was obtained against strains with MIC  $\geq 8$  mg/L.

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**Table 2.** Antipneumococcal activity of azithromycin at concentrations simulating the serum profile after a single 500 mg iv dose

Strain no.	MIC (mg/L)	AUC/MIC	24 h inocula reduction <sup>a</sup> (log <sub>10</sub> cfu/mL)	AUKC <sub>K</sub> -AUKC <sub>AZ</sub> <sup>b</sup> (cfu/mL × h)	T99.9% <sup>c</sup> (h)
1	0.5	26.9	-5.6 ± 0.58	98.0 ± 2.5	6.6 ± 1.3
2	2	6.7	-2.6 ± 0.9	68.7 ± 6.8	5.5 ± 2.1
3	8	1.7	-1.7 ± 0.6	31.7 ± 8.3	-
4	256	0.05	-0.7 ± 0.2	16.0 ± 4.3	-
Controls (antibiotic-free simulations) (strains 1-4)		0	from -0.53 to 0.01 <sup>d</sup>	-	-

<sup>a</sup>Difference between log<sub>10</sub> cfu/mL of initial inocula and log<sub>10</sub> cfu/mL at 24 h.

<sup>b</sup>Difference between the area under killing curve (AUKC) in antibiotic-free simulations (K) and azithromycin simulations (AZ).

<sup>c</sup>Time taken to obtain 99.9% reduction of initial inocula.

<sup>d</sup>Values are -0.37 ± 0.17 for strain 1, -0.50 ± 0.81 for strain 2, 0.01 ± 0.17 for strain 3 and -0.53 ± 0.4 for strain 4.

**Table 3.** Antipneumococcal activity of azithromycin at concentrations simulating the ELF profile after a single 500 mg iv dose

Strain no.	MIC (mg/L)	AUC/MIC	24 h inocula reduction <sup>a</sup> (log <sub>10</sub> cfu/mL)	AUKC <sub>K</sub> -AUKC <sub>AZ</sub> <sup>b</sup> (cfu/mL × h)	T99.9% <sup>c</sup> (h)
1	0.5	98.2	-5.7 ± 0.1	103.5 ± 7.9	6.9 ± 1.7
2	2	24.5	-4.3 ± 1.7	73.8 ± 9.4	6.3 ± 2.7
3	8	6.12	-1.3 ± 0.4	23.0 ± 3.6	-
4	256	0.20	-0.5 ± 0.5	9.1 ± 4.9	-
Controls (antibiotic-free simulations) (strains 1-4)		0	from -0.53 to 0.01 <sup>d</sup>	-	-

<sup>a</sup>Difference between log<sub>10</sub> cfu/mL of initial inocula and log<sub>10</sub> cfu/mL at 24 h.

<sup>b</sup>Difference between the area under killing curve (AUKC) in antibiotic-free simulations (K) and azithromycin simulations (AZ).

<sup>c</sup>Time taken to obtain 99.9% reduction of initial inocula.

<sup>d</sup>Values are -0.37 ± 0.17 for strain 1, -0.50 ± 0.81 for strain 2, 0.01 ± 0.17 for strain 3 and -0.53 ± 0.4 for strain 4.

From the pharmacodynamic point of view, AUC/MIC values ≥24.5 related with attainment (at around 6 h) and maintenance over 24 h of bactericidal activity (≥3 log<sub>10</sub> reduction), as occurred with strains 1 and 2 in ELF and with strain 1 in serum. Lower values were related with absence of bactericidal activity (strains 3 and 4) or with absence of maintenance over 24 h of the bactericidal activity obtained (regrowth with strain 2 in serum: T99.9% = 5.5 h, with a 24 h reduction of only 2.6 log<sub>10</sub> cfu/mL).

### Discussion

Macrolide resistance in *S. pneumoniae* in Spain is as high as 34.5%, most of the isolates (89.9%) being *erm*(B) positive,<sup>15</sup> while in the USA, resistance prevalence is 31% and most of them (70.9%) belong to the *mef*(A) genotype.<sup>16</sup> While the *erm*(B) genotype confers high-level resistance with MIC values >64 mg/L (that logically cannot be overcome by increasing antibiotic concentrations), the efflux resistance phenotype [*mef*(A) genotype] confers a much lower level of resistance. In the latter case, the question that arises is whether this low-level resistance can be overcome in body sites where the drug is concentrated.

Azithromycin is concentrated in ELF, with an AUC<sub>0-24</sub> much higher than the one in serum. In the present study experimental ELF AUC<sub>0-24</sub> was 3.64-fold higher than that in serum. Therefore if an AUC<sub>0-24</sub>/MIC of 30 is needed for bacterial eradication,<sup>17</sup>

this value would be more easily achieved by azithromycin in ELF than in serum against strains carrying the *mef*(A) gene, and with low-level resistance. Moreover, it has been suggested that concentrations at the site of infection are more predictive of antimicrobial efficacy of azithromycin against *S. pneumoniae*,<sup>8</sup> when an AUC/MIC ratio of at least 10 is achieved.<sup>7</sup> It should be considered that azithromycin antibacterial activity in ELF cannot be greatly modified by protein binding because the percentage is negligible (≤7%) when concentrations are more than 1 mg/L, and the ELF concentrations used in this study were higher than this value, as those determined in healthy adults.<sup>7</sup> These facts may explain why reports on azithromycin failure in community-acquired respiratory tract infections are uncommon, although available,<sup>18,19</sup> in areas where efflux is the prevalent phenotype of resistance.

In agreement with the previously reported value of AUC<sub>0-24</sub>/MIC needed for bacterial eradication, values around or more than 25 were associated with maintained bactericidal activity over 24 h, while regrowth or absence of bactericidal activity occurred with values <10. Although AUC<sub>0-24</sub>/MIC is the main pharmacodynamic parameter predicting efficacy for azithromycin (i.e. bactericidal activity and bacterial eradication), the pharmacokinetic profile should also be taken into account when examining the effects of similar AUC<sub>0-24</sub>/MIC against different strains.

In the present study, this can be seen when comparing the effects of the similar  $AUC_{0-24}/MIC$  (6.7 and 6.12) obtained in serum with respect to strain 2, and in ELF with respect to strain 3 (both serotype 23). In the first case (serum simulation), bactericidal activity ( $\geq 3 \log_{10}$  reduction; 99.9%) was obtained at 5.5 h against a strain with an MIC of 2 mg/L with azithromycin  $C_{max}$  levels of 3.59 mg/L achieved 1 h after the start of the experiment. In the second case (ELF simulation) bactericidal activity ( $\geq 3 \log_{10}$  reduction; 99.9%) was not obtained against a strain with an MIC of 8 mg/L with azithromycin  $C_{max}$  levels of 2.78 mg/L achieved 24 h after the start of the experiment, because levels were never supra-inhibitory. In either of the two cases, eradication and bactericidal activity were not obtained at 24 h: mean initial inocula reduction (difference between  $\log_{10}$  cfu/mL of initial inocula and  $\log_{10}$  cfu/mL at 24 h) was only 2.6 log and 1.3 log, respectively, due to low  $AUC_{0-24}/MIC$  values. Logically  $AUC_{0-24}/MIC$  values  $< 1$  (with the highly resistant strain) were only associated with growth of initial inocula without differences versus controls.

An  $AUC_{0-24}/MIC$  of 26.9 against the susceptible strain (MIC of 0.5 mg/L) seems adequate for the sustained bactericidal activity in serum. Against the other strains harbouring resistance genetic markers, azithromycin did not achieve experimental sustained bactericidal activity in serum because  $AUC_{0-24}/MIC$  values were  $< 10$  and thus are considered resistant ( $\geq 2$  mg/L) with the current CLSI breakpoints.<sup>10</sup> This is not the case with azithromycin in ELF where, with the susceptible strain and the strain with an MIC of 2 mg/L, sustained bactericidal activity was obtained because  $AUC_{0-24}/MIC$  values were around or higher than 25. From the bacterial eradication and pharmacodynamic perspective, the results of this study suggest that resistant strains with MICs of 2 mg/L, and thus intermediate-resistant strains with MICs of 1 mg/L, can be considered susceptible to the concentrations achieved in ELF after a 500 mg iv regimen. According to this, previous suggestions on breakpoint for susceptibility  $\leq 2$  mg/L based on the expected azithromycin tissue concentrations<sup>3</sup> may be adequate in the case of ELF. These suggestions should not be extrapolated to bacteraemic pneumonia where failures have been described after macrolide treatment in patients infected by M phenotype strains exhibiting erythromycin MICs as low as 4 mg/L.<sup>18</sup> This is consistent with the low  $AUC_{0-24}/MIC$  and regrowth obtained in our serum experiments with the strain with an MIC of 2 mg/L.

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## Transparency declarations

None to declare.

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