Pharmacokinetic-pharmacodynamic modeling of antibacterial activity of cephalexin on *E. coli* in presence of canine serum

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**Summary**

The aim of this study was to evaluate the activity of cephalexin and the immune response factors present in canine serum on *E. coli* ATCC 25922, using a dynamic one step dilution in vitro one compartmental model, simulating cephalexin concentrations in this biological fluid during the elimination phase after its administration of a therapeutic dose to a dog. A mathematical model was used to fit the experimental data and to simulate expected kill-curves for different single doses of cephalexin. The minimum inhibitory concentration of cephalexin was not modified by presence of serum. The kill-rate and the time required for bacterial regrowth were increased by presence of canine serum. The time period in which the bacteria were exposed to subinhibitory concentrations of cephalexin after a dose was the determining factor for the persistence of antibiotic activity and this period was prolonged when canine serum was incorporated into the in vitro assay. Those findings probably reflect the in vivo situation more closely than other in vitro assays performed in standard culture medium in absence of the immune response of the host. Based on the results of the present study is feasible to modelize the time-kill data for changing cephalexin concentrations. This is a useful method to simulate and evaluate the efficacy of different doses of this antibiotic on *E. coli*.

Keywords: Cephalexin, antibacterial activity, *E. coli*, canine serum, modeling.

**Introduction**

The quantification of the activity of antibacterial agents is the basis for the rational and safe use of them in the treatment of bacterial infectious diseases. Although, since the discovery of the antibacterial agents, the minimum inhibitory concentration (MIC) is the main pharmacodynamic (PD) parameter used for the design of antibacterial regimens [13], in the last three decades certain in vitro properties of these agents as postantibiotic effect (PAE) and the antibacterial activity of subinhibitory concentrations (sub-MIC effect) [15], have been reconsidered and have provided more information about the PD of antibiotics. Similarly, the construction of bacterial kill-curves became a useful tool to evaluate the efficacy of different concentrations of an antibiotic in function of time [14].

However, all the in vitro assays above mentioned only assess the activity of fixed concentrations of an antibiotic over time, whereas in a living organism, the total effect of certain dose depends on the large range of concentrations encountered in the patient due the pharmacokinetic (PK) processes of absorption, distribution and elimination [17,19].

An alternative in vitro model that introduces the fluctuating concentration of the antimicrobial agent is the assay to determine the postantibiotic sub-MIC effect (PAE sub-MIC). This model simulates the brief exposure of bacteria to suprainhibitory concentrations (>MIC) of antibiotics observed during the peak plasma concentration (absorption phase), followed by a more or less prolonged subinhibitory concentrations (<MIC) similar to what happens during the elimination phase of the plasma disposition curve in the in vivo scenario [13,15].

In this sense, several dynamics in vitro models have been developed for mimicking the changes in the concentrations of the antibiotic in body fluids. These models are used to evaluate the relationship between the evolution of antibiotic concentrations, and bacterial population versus time [2,4].

However, all these experimental conditions differ from what happens in a living organism, due to the absence of antibacterial...
activity of the components of the host immune system [6]. At present, the in vitro study of antibiotic activity, is intended to simulate conditions present in the in vivo scenario, evaluating the interaction between the antibiotic, bacteria and living organism (PK and immune response), because the clinical experience indicates that the success of antimicrobial therapy depends on the normal function of the patient’s defense system [10, 12].

Cephalexin is a first generation cephalosporin with a broad spectrum of activity against gram positive and gram negative bacteria. This antibiotic has excellent activity against staphylococci, streptococci, E. coli, Proteus spp., Klebsiella spp. and Pseudomonas spp. [7, 18].

The aim of this study was to investigate the in vitro antibacterial efficacy of cephalexin and canine serum (CS) against E. coli in presence of simulated canine PK profiles of cephalexin by a dynamic in vitro model based on one step dilution of culture medium, and to employ a mathematical model to describe the relationship between PK and PD data of this antibiotic for evaluating the antibacterial efficacy of different doses of cephalexin in absence and presence of CS.

**Material and Methods**

**MICROORGANISM AND ANTIBIOTIC**

One reference strain (E.coli ATCC 25922) and five clinical isolated of E. coli were tested. These strains were obtained from the Laboratory of Bacteriology, Faculty of Veterinary Sciences, Universidad Nacional del Litoral, Argentina. A standard isolate of cephalexin (Sigma-Aldrich, Chemical Company, St. Louis, USA) was used to perform this assay. This antibacterial drug exhibits time-dependent activity and based on plasma pharmacokinetic data presents a half-life of elimination of 1.5 h [1].

**CANINE SERUM**

Normal canine serum (CS) samples were obtained from six healthy dogs with no history of antimicrobial therapy within the previous 3 months. The serum was pooled and stored at -20°C until use. The pH of the pooled serum was 7.8.

**BACTERIAL INOCULUM**

The bacterial inoculum was prepared from colonies incubated on appropriate agar plates overnight. The microorganisms were suspended in sterile isotonic saline solution to a concentration equivalent to a 0.5 value in the McFarland scale (1 x 10^8 colony forming unit (CFU)/mL) measured with a turbidimeter. After that, serial dilutions were performed to obtain a final concentration of 1 x 10^6 CFU/mL.

**DETERMINATION OF MIC VALUES**

The MIC of cephalexin on the six stains of E. coli was determined by macrodilution method [3] in Muller-Hinton broth (MHB) (Britania, Buenos Aires, Argentina) at three different pH values. The growth media was prepared according to the manufacturer’s instructions and autoclaved prior to use at 121°C (15 min/1L). Broth was prepared the day before of the assay, so that it was stored in a refrigerator at approximately 7°C until use. The day of the assay the original pH of the broth (7.4) was adjusted with HCL to pH values of 6.5 and 5.5.

The MIC of cephalexin on E. coli was determined in presence of CS, so that the broth (pH 7.4) was supplemented with CS to a final concentration of 50% vol/vol (MHB/CS) with a final pH value of 7.3. Each MIC determination was performed in triplicate.

**BACTERIAL GROWTH**

Bacterial cultures in MHB (n = 6) and MHB/CS (n = 6) adjusted to 1 x 10^6 CFU/mL were incubated at 35°C during 10.5 h. Aliquots (100 µL) were removed from the incubation cultures at interval times of 1.5 h, and them were serially diluted in sterile isotonic saline solution. Aliquots (100 µL) of the final dilutions were spread in triplicate on to agar plates, and the colonies were counted after overnight incubation at 35°C.

**IN VITRO PHARMACODYNAMIC MODEL**

An in vitro PD model based on the replacement of fixed concentrations of bacteria-free culture medium at regular intervals [16] was used. This model allowed to simulate the plasma concentrations of cephalexin during the exponential elimination phase after its intravenous administration in dogs, and to investigate its antibacterial efficacy in the presence of immune response factors present in the serum.

**Procedure**

Due the time dependent antibacterial activity of cephalexin, bacterial inoculums (1 mL) in MHB and MHB/CS with a size of 1 x 10^6 CFU/mL were exposed to an antibiotic concentration equivalent to 4 x MIC respectively.

The bacterial cultures were incubated at 37°C during a period equivalent to a half-life of the drug (1.5 h). After that, with the help of a handling pipette (Boeco, Germany), 1 mL of broth without antibiotic (MHB or MHB/CS) was added to the inoculums in order to obtain by dilution a half-concentration of the antibiotic respecting the initial one. The tubes where mixed gently (Thermolyne, Mixer, Iowa, USA) and 1 mL of each tube were taken and serially diluted in sterile isotonic saline solution. Aliquots (100 µL) of the final dilutions were spread in triplicate on to agar plates, and the colonies were counted after overnight incubation at 35°C. This procedure was systematically repeated to the time equivalent to half live x 10 (15 h). The limit of quantification was 10 UFC/mL.

**Construction of growth and kill-curves**

The values of CFU/mL at each sampling time were estimated by multiplying the number of CFU/plate by the correction factor
derived from the serial dilution for each particular sample. In each growth and kill-curve, the number of viable bacteria was expressed as the average of counts made in each replicate (n = 3).

PHARMACOKINETIC-PHARMACODYNAMIC MODELING

The analysis and mathematical modeling of the growth and kill-curve data were performed with the non-linear regression software ADAPT II (BMSR, University of Southern California, USA). The data of each bacterial strain obtained in the growth curves in absence of cephalexin in MHB were fitted with the equation 1, which considers the limited available nutrients and space of the in vitro system.

\[
dN/dt = k_c \cdot \left(1 - \frac{N}{N_{\text{max}}} \right) \cdot (1 - e^{-a \cdot t})
\]

EQUATION 1

Where \(dN/dt\) is the change in the number of bacteria as a function of time, \(k_c\) (h\(^{-1}\)) in the bacterial growth rate constant in the absence of cephalexin, \(N\) (CFU/mL) the number of viable bacteria, \(N_{\text{max}}\) (CFU/mL) the maximum viable bacteria count resulting in saturation in growth, \(a\) is an adaptation rate constant for describing a delayed effect in growth.

The data obtained in the growth-curves of each bacterial strain in absence of cephalexin in MHB/CS were fitted with the equation 2.

\[
dN/dt = (k_c - k_b) \cdot \left(1 - \frac{N}{N_{\text{max}}} \right) \cdot (1 - e^{-a \cdot t})
\]

EQUATION 2

Where \(k_c\) (h\(^{-1}\)) is the bacterial growth rate constant estimated from the fitting of the growth-curves in MHB, and \(k_b\) (h\(^{-1}\)) is the bacterial kill rate constant resulting from the intrinsic bactericidal activity of canine serum. Other symbols were explained previously.

The data of the changing cephalexin concentrations and the survival bacteria profiles in MHB and MHB/CS were fitted simultaneously using equation 3.

\[
dN/dt = (k_c - k_b) \cdot (1 - e^{a \cdot t}) \cdot N
\]

EQUATION 3

where \(k_b\) is estimated as follows:

\[
k_b = \frac{k_{\text{max}} \times C^n}{EC_{50}^n + C^n}
\]

EQUATION 4

and C was simulated with the followed model:

\[
C = C_0 \cdot e^{-k_b \cdot t}
\]

EQUATION 5

where \(k_b\) is the bacterial kill-rate (h\(^{-1}\)), \(a\) is an adaptation rate constant for describing a delayed effect in growth and kill equally, \(C\) (µg/mL) is the number of viable bacteria, \(k_{\text{max}}\) (h\(^{-1}\)) is the maximum killing rate constant (maximum effect), \(EC_{50}\) (µg/mL) the concentration of the antibiotic necessary to produce 50% of the maximum effect, \(C_0\) (µg/mL) the concentration of the antibiotic at any time (t), \(n\) is the Hill coefficient or shape factor, \(C_0\) (µg/mL) is the antibiotic concentration at time zero (t\(_0\)), \(k_c\) (h\(^{-1}\)) is the elimination rate constant and t (h) is the time. Other symbols were explained previously.

QUANTIFICATION OF ACTIVITY OF CEPHALEXIN AND IMMUNE RESPONSE

The efficacy of cephalexin on six strains of E. coli was evaluated on the experimental data by descriptive and integrated PD parameters [9].

**Descriptive parameters**

- \(C_0\): initial concentration of cephalexin (t\(_0\)).
- \(C_{\text{min}}\): cephalexin concentration at time in which the minimum numbers of viable bacteria are observed.
- \(t>\text{MIC}\): time cephalexin concentration is above the MIC.
- \(t_{\text{min}}\): time in which the minimum number of viable bacteria resulting from exposure to cephalexin is observed.
- \(N_0\): initial number (t\(_0\)) of viable bacteria expressed as CFU/mL.
- \(N_{\text{min}}\): minimum numbers of viable bacteria resulting from exposure to cephalexin expressed as CFU/mL.
- \(T_E\): time shift (time interval between the zero point (t\(_0\)) when N = N\(_0\), and to the time to return to the same bacterial numbers in the regrowth phase).
- \(A\text{BE}\): additional bactericidal effect (time) calculated as: \(T_E - t_{\text{min}}\).
- \(T_{\text{SME}}\): time sub-MIC effect (time of antibacterial activity after the antibiotic concentration declined to the MIC) estimated as: \(T_E - t>\text{MIC}\).

**Integral parameters**

This kind of parameters consider both the time and the amplitude dimension of killing-time curves combining \(t\) with \(N\).

- \(\text{AUCC}_{\text{MHB}}\): area under the viable bacteria count-time curve from time zero (t\(_0\)) to the time of the last measurement (t\(_2\)) calculated by the trapezoidal from growth or kill curves in MHB.
AUCC_CS: area under the viable bacteria count-time curve from time zero (t₀) to the time of the last measurement (t_f) calculated by the trapezoidal from growth or kill curves in CS.

**Efficacy parameters**

The efficacy was expressed as percent of elimination of viable bacterial count, and a cut-off value for efficacy was set in 99.9% which is considered almost a bacterial eradication. The antibacterial efficacy of canine serum in (MHB/CS) was calculated as \[1 - \frac{(AUCC_CS/AUCC_MHB)}{100}\], and the percent of initial bacteria (N₀) eliminated at tₘᵢₙ (Eₘᵢₙ) was calculated as \[1 - \frac{(Nₘᵢₙ/N₀)}{100}\].

**PHARMACODYNAMIC SIMULATION**

The cephalexin-concentration response curves in MHB and MHB/CS were simulated using a sigmoid maximal effect model, so called Hill equation (equation 4).

The simulated bacterial time-kill curves of *E. coli* exposed to cephalexin in MHB, and MHB/CS were generated by integrating its bacteria viable count profiles (CFU/mL) in serum into the model depicted in the equation 3. The cephalexin concentration profiles after oral doses of 10, 15, 20, 25, 30 and 40 mg/kg were well described by equation 6.

\[ C = \frac{D \cdot k_a}{(k_a - k_b \cdot V_d/F)} \cdot (e^{-k_c \cdot t} - e^{-k_a \cdot t}) \]

Equation 6

Where D is the dose (µg/kg), ka (h⁻¹) is the absorption rate constant, V_d/F (mL/kg) is the volume of distribution estimated from extravascular route. Other symbols were explained previously.

The value of kₘₐₜ was fixed to 0.461 h⁻¹, which leads a half-life of 1.5 h [1], the value kₐ and V_d/F were arbitrarily fixed to 0.9 h⁻¹ and 400 mL/kg respectively in order to obtain a Cₘₐₜ value near to 29 µg/mL at 1.5 h post-administration of an oral dose of 25 mg/kg [8]. The estimated value of Cₘₐₜ at each dose was calculated using the equations 7 and 8.

\[ C_{max} = \frac{D \cdot k_a}{(k_a - k_b) \cdot V_d/F} \cdot (e^{-k_a \cdot t_{max}} - e^{-k_a \cdot t_{max}}) \]

Equation 7

\[ t_{max} = \frac{\ln k_a - \ln k_e}{k_a - k_e} \]

Equation 8

The simulations were performed by estimation of plasma concentrations with intervals of one minute, so the t>MIC was calculated by visual inspection of the simulated plasma concentration profiles.

**STATISTICAL ANALYSIS**

The PK-PD parameters are presented as mean ± standard deviation. The values obtained for each experimental group were compared by analysis of variance in order to determine significant differences (\(P < 0.05\)) between the groups.

**Results**

The estimated MIC value of cephalexin on the six strains of *E. coli* in MHB and MHB/CS was 16 µg/mL. This value was not modified by changes in the pH of MHB. The temporal evolution of bacterial mass in absence of antibiotic in MHB and MHB/CS are graphically presented in figure 1.
The viable bacterial mass after 10.5 h of incubation in MHB/CS was reduced in 59.7 ± 18.5% respecting the observed in MHB.

The real and theoretical time-concentration profiles of cephalexin obtained by the PD one-step dilution model are presented in figure 2.

The time-evolution of the bacterial mass of E. coli, in function of cephalexin concentrations simulating a first order of elimination in MHB and MHB/CS are presented in figure 3.

The lowest viable bacterial count was observed at 4.5 h in MHB and MHB/CS. The lowest percent of reduction of viable bacteria respecting the initial inoculums size was observed in MHB (80.7 ± 5.16%), while the observed in MHB/CS was 91.1 ± 6.43%. The shift time (T_E) observed in MHB was 7.20 ± 0.62 h, while in MHB/CS this value was extended to 8.46 ± 0.87 h. The additional bactericidal effect (ABE) in MHB and MHB/CS were 2.70 ± 0.62 h and 3.96 ± 0.87 h respectively, and the time of antibacterial activity of subinhibitory concentrations (TSME) were 4.20 ± 0.62 h in MHB and 5.46 ± 0.87 h in MHB/CS (Table I).

The fitting of the experimental data of growth bacterial curves of E. coli in MHB and MHB/CS are presented in the figure 1. The bacterial growth-rate (k_c) estimated from data of growth curves in MHB was 1.44 h⁻¹ while in MHB/CS this value was 1.05 h⁻¹ (table II).

The fit of the experimental data of kill-curves of E. coli in function of time in presence of cephalexin in MHB and MHB/CS, are presented in figure 4 and the fitting parameters are presented in table II.

The estimated PD parameters of the antibacterial activity of cephalexin presented differences between the culture medium employed (MHB and MHB/CS). The antibiotic efficacy was increased in presence of CS where the estimated k_max value was 8.98 h⁻¹ in MHB/CS respect the estimated value in MHB (4.40 h⁻¹). A high value of EC_50 was observed in MHB/CS (17.3 µg/mL) respect the observed in MHB (9.63 µg/mL), and a 95% of the maximal response was obtained in MHB/CS with a concentration of 54.5 µg/mL, whereas in MHB this value was 15.23 µg/mL (Table II).

The simulated profiles of plasma concentration of cephalexin after oral administration of several doses to dogs are presented in figure 5.

**FIGURE 2:** Time concentration profile of cephalexin simulated by the dynamic one-compartmental in vitro model with an initial concentration of 64 µg/mL. The solid line represents the real concentrations generated by the model, the dotted line represents the theoretical time-concentration profile simulating an exponential elimination phase with a half-life of 1.5 h, and the dropped line is the MIC value of cephalexin on E. coli (16 µg/mL).

**FIGURE 3:** Time-kill curves of E. coli (n = 6) in MHB (○) and MHB/CS (○) over time in function of cephalexin concentrations simulating an exponential elimination phase with a half-life of 1.5 h by the dynamic one-compartmental in vitro model. The values of viable bacteria are expressed as CFU/mL and are presented as mean ± standard deviation.

**TABLE I:** Descriptive and efficacy parameters for evaluating the antibacterial activity of cephalexin on E. coli (n = 6) in MHB and MHB/CS. The values are expressed as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MHB</th>
<th>MHB/CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>t &gt; MIC (h)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cmin (µg/mL)</td>
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<td>8</td>
</tr>
<tr>
<td>t_min (h)</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Et_min (%)</td>
<td>80.7 ± 5.16*</td>
<td>91.1 ± 6.43*</td>
</tr>
<tr>
<td>T_E (h)</td>
<td>7.20 ± 0.62*</td>
<td>8.46 ± 0.87*</td>
</tr>
<tr>
<td>ABE (h)</td>
<td>2.70 ± 0.62*</td>
<td>3.96 ± 0.87*</td>
</tr>
<tr>
<td>TSME (h)</td>
<td>4.20 ± 0.62*</td>
<td>5.46 ± 0.87*</td>
</tr>
</tbody>
</table>

t > MIC: time at which cephalexin concentration is above the MIC. 
C_min: cephalexin concentration at time in which the minimum numbers of viable bacteria are observed. 
t_min: time in which the minimum number of viable bacteria resulting from exposure to cephalexin is observed. 
Et_min: efficacy expressed as the percent of N_0 eliminated at t_min calculated as 1-(N_min/N_0) x 100. 
T_E: time interval between the zero point (t_0) when N = N_0 and to the time to return to the same bacterial numbers in the regrowth phase (regrowth bacterial time). 
ABE: additional bactericidal effect (time) calculated as RBT-t_min. 
TSME: time sub-MIC effect. 
(*) groups differs significantly (P < 0.05).
The simulated profiles of viable bacterial count of *E. coli* over time exposed to cephalexin after oral administration of several doses to dogs are presented in figure 6.

The values of the indices of antibacterial efficacy obtained by effect-plasma concentration simulation are presented in table III, and the relationship between indices of antibacterial efficacy of cephalexin at different doses vs. Et_{min}, t>MIC, TSME and TE are presented in figure 7.

The relationship between dose and Et_{min} value is nonlinear in both MHB and MHB/CS. In this study, plots of Et_{min} versus doses of cephalexin obtained in MHB and MHB/CS show a rapid rise toward an asymptote (Figure 7A), however only the dose of 40 mg/kg in presence of canine serum was able to eliminate 99.9% of the initial viable bacterial count (Figure 6F and Table III). On other hand increased doses resulted in increased t>MIC values (table III, figure 7B). In the same way increased doses, result in increased T_E and T_{SME} values (Table III and Figure 7C-D).

**Discussion**

The dose-response relationship of antibiotics are often not well known, because of the pure antibiotic effect observed *in vitro* does not consider other factors determining the response
FIGURE 6: Simulation of the antibacterial activity of cephalexin on *E. coli* after its extravascular administration to dogs at doses of 10 mg/kg (A), 15 mg/kg (B), 20 mg/kg (C), 25 mg/kg (D), 30 mg/kg (E) and 40 mg/kg (F). The time-evolution of viable bacterial count in MHB (thick line) and in MHB/CS (dotted line) are expressed as log10 of CFU/mL. Visual inspection of the figure shows that only a cephalexin dose of 40 mg/kg in presence of canine serum achieves more than 99.9% reduction in viable bacterial count.

FIGURE 7: Antibacterial activity of cephalexin on *E. coli* in MHB and MHB/CS. Effect of increased doses on $E_{t_{\text{min}}}$ (A), $t_{>\text{MIC}}$ (B), $T_{S\text{ME}}$ (C), and $T_{E}$ (D). The values were generated by simulation of effect-concentration profiles.
of the antibacterial treatment in a patient. These factors are interactions between host (PK and immune response), microorganism and treatment (drug).

The knowledge of the interaction between antibiotic and microorganism is not sufficient because it is accepted that the most important factor for the outcome of an infection is the interaction between patient and causative microorganism, so it is important to realize that the patient must cure himself, since antibiotic treatment only helps indirectly by combating the microorganism [11].

Thus the PK, PD and the immune response of the host should be considered in the development and prediction of the efficacy of antibacterial therapy. In that way, dynamic models simulating antibiotic PK have been proven to be useful tools for investigating the in vitro antibiotic activity. Biological fluid as serum can be incorporated into the models in order to evaluate the interaction between antibiotic and some of the components of host defense. The results obtained in this assay provide information about the synergistic antibacterial activity of cephalexin and the immune response factors present in CS on six strains of *E. coli*.

Cephalexin is a weak acid, and although primarily dissociated as an acid (pKₐ 5.2), is amphoteric and also has a pKₐ value of 7.3 [5]. These features could explain why the in vitro activity (MIC) was not modified by the pH of the culture medium within a range of 5.5 to 7.4.

On other hand, our results shown that the MIC was not modified by the presence of CS, which could be interpreted as a lack of antibacterial activity of this biological fluid on the microorganism. Moreover, it should be kept in mind that the MIC constitutes a static PD parameter that does not provide information about the bactericidal kill-rate. By definition, the MIC constitutes a static PD parameter that does not provide information about the bactericidal kill-rate. By definition, the MIC is an antibiotic concentration that prevents the visible bacterial growth detected by unaided eye determined in two-fold dilution step. In consequence MIC is a point measurement with poor precision that does not allow discriminate between bactericidal or bacteriostatic activity, or simply a delay in the rate of bacterial growth. In view of the above exposed, the lack of effect of CS on MIC values, does not exclude the existence of antibacterial activity due the immune response factors present in this biological fluid.

The evaluation of the intrinsic antibacterial activity of CS was performed from the results obtained of the growth-bacterial curves (Figure 1). The evolution of the bacterial population should be interpreted as the result of two competitive processes: a continuous growth of a portion of bacteria population and the death of another portion. The apparent bacterial growth-rate constant (kg) estimated by fitting of the experimental data of growth-curves (1.44 h⁻¹), is the result of the difference between a real growth-rate constant (kg) and a real death-rate constant (kd) as expressed as follows (kg = kg - kd). The presence of CS in the culture medium increased the theoretical kd value, resulting in an apparent growth-rate constant of 1.05 h⁻¹. As result of the delay in the growth process in MHB/CS, the bacterial mass of *E. coli* was reduced in 59.7 ± 18.5% respecting the bacterial mass quantified in the standard culture medium, in which the lack of host defenses represent conditions that resembles to that encountered in an immunodeficient patient [20].

The reduction of viable bacteria in presence of CS can be attributed to the activity of the complement system, which is a vital part of the body’s immune system providing a highly effective means for the destruction of invading microorganisms. Bacteria can be directly lysed by complement proteins, which are activated by three pathways: the classical pathway, the alternative pathway and the lectin pathway, all of which lead to the formation of the cytolytic membrane attack complex.

The results obtained from the kill-curves shown that the minimum viable bacterial number was observed at 4.5 h and the bacterial regrowth was observed at a cephalexin concentration of 8 μg/mL in both MHB and MHB/CS culture medium (Table I), moreover the efficacy of cephalexin was increased in presence of CS in which the value of Tₘᵢₙ was significantly greater (91.1 ± 6.43%) than the observed value in MHB (80.7 ± 5.16%).

An important parameter considered into our analysis was the Tₘₑₐ, this parameter allowed us to detect differences in the evolution of antibacterial activity of cephalexin in both culture media, the value obtained in MHB/CS (8.46 ± 0.87 h) being greater than the observed value in MHB (7.20 ± 0.62 h).

The most important difference between Tₘₑₐ values were encountered in Tₛₑₐₐ values, being 5.46 ± 0.87 h in presence of

<table>
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<tr>
<th>Dose (mg/kg)</th>
<th>Cₘₚₓ (μg/mL)</th>
<th>Cₘₚₓ/MIC MHB</th>
<th>Cₘₚₓ/MIC MHB/CS</th>
<th>Etₘᵢₙ (%) MHB</th>
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<th>t&gt;MIC (hours) MHB</th>
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<td>49.49</td>
<td>3.09</td>
<td>99.08</td>
<td>99.93</td>
<td>5.10</td>
<td>7.20</td>
<td>12.90</td>
<td>12.30</td>
<td>18.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cₘₚₓ: maximal antibiotic concentration estimated by pharmacokinetic simulation.
Other the symbols were explained previously

**TABLE III:** Estimated values of pharmacokinetic data and indices of antibacterial efficacy of cephalexin on *E. coli* (n = 6) obtained by simulation of the effect-concentration relationship of different doses administered to dogs by oral route in presence and absence of canine serum.
CS and 4.20 ± 0.62 in MHB (Table I). This means that over 50% of the value of $T_E$ is due to the activity of subinhibitory concentrations of the antibiotic. Indeed, the value of $T_{SME}$ observed in MHB represents 64.4 ± 6.75% of the value of $T_E$, while in MHB/CS this percentage is 73.6 ± 3.45%.

The effect of subinhibitory concentrations of antibiotics has been reported extensively, and they are capable of producing on bacteria morphological and functional alterations that retard its growth. The increase in $T_{SME}$ observed in the presence of CS clearly shows that the interaction between serum bactericidal activity and intrinsic antibiotic activity increased antibacterial efficacy in terms of prolong the $T_E$.

For antibiotics like for any other drugs, the relationship between effect and concentration is non linear, and can be characterized by at least two parameters, i.e. the maximum obtainable effect or efficacy ($E_{max}$) and the concentration at which the half of efficacy is attained ($E_{50}$). This relationship is often expressed by the so called Hill equation (equation 4).

The nonlinearity of the concentration-response of cephalaxin can be explained by the time dependent activity of the $\beta$-lactam antibiotics, for which the maximum killing rate ($k_{max}$) occurs at low multiples of MIC. The presence of CS modified the antibacterial activity of cephalaxin on $E. coli$ in terms of bactericidal kill-rate ($k_b$). The inspection of the table II indicated that in standard broth, the kill-rate constant observed with a cephalaxin concentration higher than 20 µg/mL reached a near maximal value (4.40 h$^{-1}$), and the estimated value of the slope term (6.42) greater than 4 is reflective of an all-or-none type of response. On other hand, in MHB/CS, the $k_b$ values increased gradually to reach 95% of the maximal value (8.98 h$^{-1}$) at cephalaxin concentration of 54.5 µg/mL (Table II).

These results indicated that the immune factors present in CS not only increased the maximal response in terms of $k_{max}$, but also increased the activity of cephalaxin concentrations beyond of 20 µg/mL.

On other hand, the activity of the immune factors of the host present in serum determined that the antibacterial effectiveness of cephalaxin observed in MHB/CS expressed in terms of $T_{SME}$ and $T_E$ was greater than the observed in MHB (Table III, Figures 7C and 7D).

From the results obtained in the simulation study of the dose-effect relationship, some considerations regarding the activity of this antibiotic can be made. Like all the $\beta$-lactams, the effectiveness of cephalaxin is determined by the time of exposure of the bacteria at suprainhibitory concentrations. Therefore, it is assumed that increased concentrations resulted from increased from doses do not improve its efficacy in terms of bacterial killing rate and reduction of viable bacteria.

Our results shown that the maximum efficacy value $E_{min}$ (99.93%) was reached with a dose of 40 mg/kg ($C_{max}$/MIC of 3.09) only in MHB/CS (Table III and Figure 7A).

However, in the simulation study of the dose-effect relationship was observed that the increasing the dose, the greater the time of exposure of bacteria at suprainhibitory concentrations (>MIC) of cephalaxin (Table III and Figure 5 and 7B), and as a result, the time at which the bacteria are exposed to subinhibitory concentrations ($T_{SME}$) was also increased.

In view of the results obtained, it is clear that in a linear pharmacokinetic system with first order processes of absorption an elimination in absence of a flip-flop phenomenon, the main factor able to controlling the duration of $T_E$ (>MIC + $T_{SME}$) is the kinetic disposition of cephalaxin, which is determining by the dose administered.

**Conclusion**

In this study we have evaluated the activity of cephalaxin on $E. coli$ in a culture medium that simulates the situation of an immunodeficient patient (MHB) or an immunocompetent patient (MHB/CS).

The results obtained showed that the ($T_E$) is a PD measurement of antimicrobial activity which influence optimal dosage interval, and its duration is controlling by the kinetic disposition of the antibiotics. Since a period with sub-inhibitory concentrations will often exist between the doses when intermittent dosing of antibiotic is used, the $T_{SME}$ is the determining factor for the persistence activity of antibiotics which exhibits time dependent activity. In linear pharmacokinetic systems the increasing the doses, the increasing the values of >MIC and $T_{SME}$ values, as consequence the $T_E$ increasing also.

An increase of $T_{SME}$ was obtained when the intrinsic antibacterial activity of CS was incorporated to the in vitro assay, and this finding probably reflects the in vivo situation more closely than other in vitro assays performed in standard culture medium in absence of the immune response of the host.

These results support the conclusion that in view of the observed relationship between dose and $T_E$ (>MIC and $T_{SME}$), and the participation of the host defense factors, the dosing schedule of cephalaxin could be modified ie: increasing the dose and reducing the number of administrations without affecting the efficacy of antibiotic treatment.

It should be noted that this study has not assessed the activity of the inflammatory response and the activity of phagocytic cells, so it should be keep in mind that the real activity in the presence of the complete immune response of the host should be greater than the observed in this trial, therefore future clinical trials are needed to corroborate the above. However it is clear that the recommended dose of a $\beta$-lactams should be reviewed to optimize the design of therapeutic regimens of this kind of antibacterial agents.

Based on the results of the present study the approach of combining in vitro time-kill data with existing in vivo PK data can be used for devising optimal treatments strategies and to define optimal antibiotic regimens.

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