Effect of the bovine serum and persister cells on the efficacy of enrofloxacin and ciprofloxacin against a strain of *Escherichia coli*

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SUMMARY

The *in vitro* activity of enrofloxacin and ciprofloxacin against *Escherichia coli* ATCC 25922 by time-kill assays was evaluated, and the additional antibacterial activity of bovine serum and the effect of the persister cells on the bacterial kinetic elimination were also evaluated. The assays were performed using combined concentrations of enrofloxacin and ciprofloxacin. The proportion between both drugs was the same as those found in bovine plasma following the administration of a therapeutic dose of enrofloxacin. Even in the presence of bovine serum, supra-inhibitory concentrations of enrofloxacin and ciprofloxacin did not eliminate persister cells and resulted in similar bacterial elimination profiles and similar efficacies. Some profiles were fitted with two exponential model and some were fitted by a three exponential model. The first and the second terms of the model were interpreted as a mathematical expression of the fast bacterial elimination kinetics of antibiotic-susceptible bacterial sub-populations, and the third exponential term was associated to the slow elimination kinetics of the persister cells sub-population. From the results obtained in this study, it is possible to infer that the fast elimination of sensible cells and slow elimination of persister cells are two simultaneous consequences of the activity of enrofloxacin and ciprofloxacin which cannot be evaluated separately. On the contrary, they constitute two ways of the same antibacterial activity, and their efficacies depended on their high concentration and the time of exposure to them.

Keywords: Enrofloxacin, ciprofloxacin, *Escherichia coli*, persister cells, efficacy, pharmacodynamics

RÉSUMÉ

Effet du sérum bovin et des cellules persisters sur l’efficacité de l’enrofloxacine et de la ciprofloxacine contre une souche d’*Escherichia coli*

L’activité *in vitro* de l’enrofloxacine et la ciprofloxacine sur *Escherichia coli* ATCC 25922 a été évaluée par des études de cinétiques bactéricides. L’activité antibactérienne de sérum de bovin et l’effet des bactéries persistantes sur la cinétique de bactéricidie a également été évaluée. Les études ont été effectuées en utilisant des concentrations combinées de l’enrofloxacine et la ciprofloxacine. Le rapport du médicament était le même que celui trouvé dans le serum de bovin après l’administration d’une dose thérapeutique de l’enrofloxacine. Même en présence de sérum de bovin, des concentrations élevées de l’enrofloxacine et la ciprofloxacine, ont été incapables d’éliminer les bactéries persistantes, et ont donné lieu à des profils semblables de clairance bactérienne, qui ont présenté des efficacités similaires. Certains profils ont été définis avec une expression triexponentielle, et d’autres ont été définis avec une expression biaxponentielle. Le premier et le second terme exponentiel ont été interprétés comme des expressions mathématiques de la cinétique d’élimination rapide d’une sous-population bactérienne sensible, et le troisième terme exponentiel a été associé à une cinétique d’élimination lente d’une sous-population de bactéries persistantes. A partir des résultats obtenus dans cette étude, nous pouvons en déduire que l’élimination rapide des bactéries sensibles et l’élimination lente des bactéries persistantes sont deux effets simultanés de l’activité de l’enrofloxacine et la ciprofloxacine et qui ne peut être évaluée séparément. Au contraire, ce sont deux modes de la même activité antibactérienne de ces médicaments et leur efficacité dépend de leur concentration élevée et le temps d’exposition à eux.

Mots-clés : Enrofloxacine, ciprofloxacine, *Escherichia coli*, cellules persisters, efficacité, pharmacodynamique

Introduction

It is clear that the course of an infection, and therefore the success of antibiotic treatment, depends on a series of host factors, including age, state of health, immunological status and even genetics. After all, antibiotics have only a supportive role in the control and clearance of bacterial infections [19].

By far, the most important factor for the outcome of an infection is the interaction between patient and causative microorganism, since antibiotic treatment only helps indirectly by combating the microorganism [4, 23]. Clinical experience indicates that the success of antimicrobial therapy depends on the normal function of the patient’s defense system [18, 24]. Therefore, to establish optimum therapeutic regimens, a thorough knowledge of the interaction between antibiotic and microorganism is not sufficient [23].

Susceptibility to the serum bactericidal system is a widespread characteristic of Gram-negative bacteria. This phenomenon has been widely noted and studied since the late 1800s [32] and has been shown to be complement mediated [16, 29].

In addition to complement, lysozyme, calcium (Ca²⁺) and magnesium (Mg²⁺) are important components of extracellular fluids that are required for killing Gram-negative bacteria. Calcium and magnesium ions are essential for initiation of the classical complement sequence [25]. It has been established that immunoglobulins as IgG and
IgM in the presence of complement were able to promote killing of some Gram-negative bacteria in vitro [26], but immunoglobulin-M fraction was proven to be bactericidal [30].

Consequently, the search for new strategies for the treatment of bacterial diseases should consider the study of the interaction between microbiology, pharmacology and immunology by using in vitro models which simulate conditions present in the in vivo scenario to evaluate the interaction between bacteria, antibiotics and immune factors present in the living organism, and thus generate information to be used for the design and optimization of dosage regimens.

In that respect, in vitro assays performed by incorporating host serum into the culture medium allow us to simulate environmental conditions that approximate those found in an in vivo assay.

Since the early days of the antibiotic era, it has been known that when bacteria in an actively dividing culture have sufficient resources for metabolism and growth, bactericidal antibiotics cannot kill all the bacteria [3]. As time proceeds, the rate of killing declines and substantial fractions of the bacterial population survive their encounter with these drugs. This fraction of the bacterial population has been called 'persisters' [1, 3].

Persisters are slow growing, non-growing or dormant cells that constitute a small fraction of the cells that remain in the bulk of any bacterial population. Their physiological status allows them to escape the lethal action of antibiotics without expressing an antibiotic resistance mechanism being phenotypic variants of regular cells.

It has been proposed that the persister cells are the result of a policy of survival for the entire population, because in changing environments like those subjected to lethal antibiotic concentrations [2] or other stressor factors such as variations in temperature, acidic pH, oxidative stress or nutrient-limiting conditions [17, 22].

These persister cells can save the population from extinction during the time of stress and ensures the survival of the population as a whole in the presence of lethal concentrations of antibiotics.

Enrofloxacin (EFX) is a fluoroquinolone (FQ) licensed for use in cattle in many countries throughout the world. Enrofloxacin is known to be partially metabolized to ciprofloxacin (CFX) in cattle, and CFX achieves ≈70% of the concentration of the parent drug in blood [11].

It is accepted that EFX y CFX are antibiotics which present concentration-dependent mode of action, this means that the higher the drug concentration, the grater the rate and extent of bactericidal activity [10]. Notwithstanding, has been reported that EFX concentrations comprised between 1 x MIC and 32 x MIC, gave rise to similar bacterial elimination profiles and similar efficacies [6]. Similar results were observed for marbofloxacin concentrations comprised between 4 x MIC and 32 x MIC and between 2 x MIC and 16 x MIC for sarafloxacin [13, 35] against Escherichia coli respectively. On the other hand similar results for norfloxacin against Staphylococcus aureus were reported [14].

An explanation for this phenomenon could be that from a threshold antibiotic concentration, the concentration-dependent killing leads the selection of a small sub-population of persister cells which neither die nor growth in the presence of antibiotics and present a slow elimination rate which is not affected by the increased antibiotic concentrations.

Considering that after administration of a therapeutic dose of EFX to bovine, plasma concentrations of EFX and its active metabolite CFX are obtained, the present work aimed at evaluate: (i) the antibacterial activity of combined concentrations of EFX and CFX (EFX-CFX) against Escherichia coli by time-kill assays, (ii) the additional antibacterial activity of bovine serum (BS) on the antibacterial activity of EFX-CFX and (iii) the effect of the persister sub-population of Escherichia coli on the bacterial kinetic elimination.

Materials and methods

MICROORGANISM AND ANTIBIOTIC

A strain of E. coli (ATCC 25922) and reference standards of EFX and CFX (Sigma-Aldrich, Chemical Company, St. Louis, USA) were selected to perform this assay.

BOVINE (BOS TAURUS) SERUM

Normal BS samples were obtained from six healthy adult Holstein calves with no history of antimicrobial therapy within the previous 3 months. The serum was pooled and stored at -20°C prior to use.

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 10⁸ colony forming units [CFU]/mL) measured with a turbidimeter. After that, serial dilutions were performed to obtain a final concentration of 0.5 x 10⁶ CFU/mL.

determination of MIC and MBC VALUES

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of EFX and CFX on E. coli (ATCC 25922) were determined by macrodilution

PERSISTER CELLS AND THE EFFICACY OF ENROFLOXACIN AND CIPROFLOXACIN

10^6 cfu/mL were exposed to constant
MIC. 13.46 x MIC, 26.92 x MIC, 53.85 x MIC, and
0.21 x MIC, 0.42 x MIC, 0.84 x MIC, 1.68 x MIC, 3.37 x MIC,
6.73 x MIC, 13.46 x MIC, 26.92 x MIC, 53.85 x MIC, and
then incubated at 35°C during 24 h. Aliquots (100 µL) were
removed from the incubation cultures at 0, 1, 2, 3.5, 5, 10 and
24 hours and then were serially diluted in sterile isotonic
saline solution. Aliquots (100 µL) of the final dilutions were
spread in duplicate onto agar plates, and the colonies were
counted after overnight incubation at 35°C.

TIME-GROWTH CURVES

Bacterial cultures in MHB and MHB-BS adjusted to 0.5
x 10^6 cfu/mL were incubated at 35°C for 24 h. Aliquots (100
µL) were removed from the incubation cultures at 0, 1, 2, 3.5,
5, 10 and 24 h and then were serially diluted in sterile isotonic
saline solution. Aliquots (100 µL) of the final dilutions were
spread in duplicate onto agar plates, and the colonies were
counted after overnight incubation at 35°C.

TIME-KILL CURVES OF EFX AND CFX

Time-kill curves of E. coli (ATCC 25922) were performed
for (i) EFX and (ii) CFX. The drug concentrations were
selected taking as a reference the MIC of each antibiotic
determined in MHB. For each time-kill curve, bacterial
cultures adjusted to 0.5 x 10^6 cfu/mL were exposed in MHB
to constant concentrations of EFX or CFX equivalent to 0.25
x MIC, 0.5 x MIC, 1 x MIC, 4 x MIC, 8 x MIC, 16 x MIC and
64 x MIC, and then incubated at 35°C during 24 h. Aliquots
(100 µL) were removed from the incubation cultures at 0, 1,
2, 3.5, 5, 10 and 24 h and then were serially diluted in sterile
isotonic saline solution. Agar plates were inoculated with 100
µL of the final dilutions by duplicate, and the colonies were
counted after overnight incubation at 35°C.

TIME-KILL CURVES OF (EFX+CFX) AND (EFX+CFX)BS

Time-kill curves of E. coli (ATCC 25922) were performed
for (i) combined concentrations of enrofloxacin and
ciprofloxacin (EFX+CFX) and (ii) combined concentrations
of EFX and CFX in the presence of bovine serum (EFX+CFX)
BS.

The drug concentrations were selected taking as a
reference the MIC of each antibiotic. The EFX concentrations
used were 0.0027, 0.0055, 0.0109, 0.022, 0.044, 0.088, 0.18,
0.35 and 0.70 µg/mL. The concentrations of CFX were
fixed at 70% of the concentrations of EFX [11], being 0.0019,
0.0038, 0.0077, 0.015, 0.031, 0.061, 0.12, 0.25, and 0.50 µg/
ML. The (EFX+CFX) concentrations were expressed as the
sum of the multiples of the estimated values of MIC [µg/ML/
MIC] of each antibiotic, so bacterial cultures of E. coli ATCC
25922 adjusted to 0.5 x 10^6 cfu/mL were exposed to constant
(EFX+CFX) and (EFX+CFX)BS concentrations equivalent to
0.21 x MIC, 0.42 x MIC, 0.84 x MIC, 1.68 x MIC, 3.37 x MIC,
6.73 x MIC, 13.46 x MIC, 26.92 x MIC, 53.85 x MIC, and

CONSTRUCTION OF TIME-GROWTH AND TIME-KILL
CURVES

The bacterial concentration values in 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. The limit of detection
was 10 cfu/mL. In each time-growth and time-kill curve, the
number of bacteria was expressed as the average of counts
made in duplicate. In order to facilitate the construction
and interpretation of the curves, the bacterial counts were
expressed as log_{10} cfu/mL.

EFFICACY BREAKPOINTS

Three breakpoints for descriptive parameters were
considered for evaluating the efficacy (E) of EFX, CFX,
(EFX+CFX) and (EFX+CFX)BS concentrations against E. coli
(ATCC 25922): (i) bacteriostasis was defined as no change in
the initial bacterial count (E = 0) after 5 h incubation, (ii)
bactericidal action was defined as 3 log_{10} reduction of the
initial bacterial count (E = 99.9%) after 24 h incubation and
(iii) virtual bacterial eradication was defined as 4 log_{10}
reduction of the initial bacterial count (E = 99.99%) after 24
h incubation [31].

DETERMINATION OF MIC VALUES OF SURVIVING BACTERIA

The MIC of EFX and CFX on surviving bacteria after 24
h of exposure to EFX, CFX, (EFX+CFX) and (EFX+CFX)BS
was determined by macrodilution method [7] in MHB.

PHARMACODYNAMIC MODELING

The analysis and mathematical modeling of the time-
growth curves and time-kill curves were performed by
non-linear regression analysis using the software ADAPT II
(BMSR, University of Southern California, USA).

The parameters were calculated directly from viable
counts (cfu/mL) without log transformation. Weightings
used for the xth measured viable count were 1, 1/x and 1/
x^2, being x the bacterial count expressed as cfu/mL. Plots
of weighted residuals were inspected and the best weighting
scheme was selected based on the scatter and random
distribution of residuals about the abscissa.

The relationship between concentrations of EFX, CFX,
(EFX+CFX) and (EFX+CFX)BS and log_{10} difference between
bacterial count (cfu/mL) after 24 h incubation and the initial
inoculums bacterial count of E. coli ATCC 25922 were
described using the Hill (sigmoidal \( E_{max} \) ) model, the basic equation for which is:

\[
E = E_0 + \frac{E_{max} \cdot C^y}{EC_{50}^y + C^y}
\]

where \( E \) is the antibacterial effect measured as the change in the log_{10} difference between bacterial count (cfu/mL) after 24 h incubation and the initial inoculum bacterial count; \( E_{max} \) is the maximal efficacy obtainable; \( E_0 \) is the log_{10} difference between bacterial count (cfu/mL) after 24 h incubation and the initial inoculum bacterial count in the absence of antibiotics; \( C \) is the antibiotic concentration expressed as multiples of the MIC [(µg/mL)/MIC] for EFX and CFX, and the sum of multiples of MIC for (EFX+CFX) and (EFX+CFX)BS; \( EC_{50} \) is the antibiotic concentration expressed as described above producing 50% of the \( E_{max} \) and \( y \) is the Hill coefficient which represents the slope of the concentration–effect relationship.

From the parameters estimated by the \( E_{max} \) model and considering the efficacy breakpoints, the theoretical concentrations capable of obtaining: (i) bacteriostatic effect, which is equivalent to a estimated MIC (MIC\(_E\)), (ii) bactericidal effect, which is equivalent to a estimated MBC (MBC\(_E\)) and (iii) bacterial eradication expressed as minimum bacterial eradication concentration (MEC\(_E\)) were calculated with the following equation derived from the Hill model:

\[
C = \left[ \frac{\left(E - E_0\right) \cdot CE_{50}^y}{E_{max} + E_0 - E} \right]^{\frac{1}{y}}
\]

Where \( C \) is the theoretical antibiotic concentration expressed as explained above, capable of obtaining a certain antibacterial efficacy; \( E \) is the expected efficacy as bacteriostatic, bactericidal or bacterial eradication. Other symbols were explained previously.

The bacterial count data obtained in the time-growth curves in the absence and in the presence of BS were fitted with the following equation, which considers the limited available nutrients and space of the in vitro system.

\[
\frac{dN}{dt} = k_G \cdot (1 - e^{zt}) \cdot N
\]

Where \( dN/dt \) is the change in the number of bacteria as a function of time; \( k_G \) (h\(^{-1}\)) is the bacterial growth rate constant in the absence and in the presence of BS; \( z \) is an adaptation rate constant for describing a delayed effect in growth; \( t \) is the time and \( N \) is the number of viable bacteria expressed as cfu/mL.

From the estimated values \( k_G \), the half-time (h) of bacterial elimination rate related to the antibiotic activity \( (t_{1/2b}) \) were estimated as ln\(_2\)/\( k_G \).

**Results**

**MIC AND MBC VALUES OF EFX AND CFX**

The estimated MIC values of EFX and CFX against \( E. coli \) (ATCC 25922) were 0.0312 and 0.0156 µg/mL respectively and the estimated MBC values of EFX and CFX were 0.0624 and 0.0156 µg/mL respectively.

**ANTIBACTERIAL ACTIVITY OF EFX AND CFX**

The 24 h evolution of viable cell count as a function of several concentrations of EFX and CFX are presented in Figure 1A-B respectively.
PERSISTER CELLS AND THE EFFICACY OF ENROFLOXACIN AND CIPROFLOXACIN

The estimated slope values ($y$) of EFX and CFX were 3.06 and 5.10 respectively, which shows an all-or-nothing response. The potency expressed as $EC_{50}$ value was higher for CFX than the observed for EFX then bacteriostatic and bactericidal effects were attained by CFX at lower multiples of MIC than EFX. Bacterial eradication was attained solely by CFX at multiples of MIC no more than twice what was required to attain the bacteriostatic effect (Table 2).

After 24 h of exposure, CFX concentrations equivalent to 2, 4, 8 and 32 x MIC reached virtual bacterial eradication effect (Figure 1 B), while for EFX, the same multiples of MIC reached only a bactericidal effect (Figure 1 A).

Plots of the relationship between EFX and CFX concentrations and difference between bacterial count (cfu/mL) after 24 h incubation and the initial inoculums bacterial count are present in Figure 2 A and B. Pharmacodynamic parameters resulting from fitting the experimental data with the sigmoid $E_{\text{max}}$ model are presented in Table 1.

The estimated slope values ($y$) of EFX and CFX were 3.06 and 5.10 respectively, which shows an all-or-nothing response. The potency expressed as $EC_{50}$ value was higher for CFX than the observed for EFX then bacteriostatic and bactericidal effects were attained by CFX at lower multiples of MIC than EFX. Bacterial eradication was attained solely by CFX at multiples of MIC no more than twice what was required to attain the bacteriostatic effect (Table 2).

Table 1: Pharmacodynamic parameters estimated by fitting of the relationship between concentrations of (A) EFX, (B) CFX, (C) (EFX+CFX) and (D) (EFX+CFX)BS. In A and B the drug concentration was expressed as the multiples of the estimated values of MIC of each antibiotic. In C and D, the concentrations of CFX were fixed at 70% of the concentrations of EFX and their mixed concentrations were expressed as the sum of the multiples of the estimated values of MIC [µg/mL/MIC] of each antibiotic. Each experimental data point represent the mean cfu/mL (n=2). The dotted lines indicate (a) bactericidal effect (a 3 log$_{10}$ reduction) and (b) virtual bacterial eradication (a 4 log$_{10}$ reduction).

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>EFX</th>
<th>CFX</th>
<th>(EFX+CFX)</th>
<th>(EFX+CFX)BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_0$ (cfu/mL)</td>
<td>3.20</td>
<td>2.83</td>
<td>2.80</td>
<td>3.80</td>
</tr>
<tr>
<td>$E_{max}$ (cfu/mL)</td>
<td>-7.07</td>
<td>-7.22</td>
<td>-7.47</td>
<td>-8.11</td>
</tr>
<tr>
<td>Efficacy (log$_{10}$ reduction)</td>
<td>-3.87</td>
<td>-4.39</td>
<td>-4.67</td>
<td>-4.31</td>
</tr>
<tr>
<td>$EC_{50}$ [µg/mL/MIC]</td>
<td>0.95</td>
<td>0.72</td>
<td>0.60*</td>
<td>0.38*</td>
</tr>
<tr>
<td>$γ$ (Hill coefficient)</td>
<td>3.06</td>
<td>5.10</td>
<td>5.47</td>
<td>3.91</td>
</tr>
<tr>
<td>(µg/mL)/MIC for Efficacy = 0</td>
<td>0.89</td>
<td>0.66</td>
<td>0.54*</td>
<td>0.37*</td>
</tr>
<tr>
<td>(µg/mL)/MIC for -3 log$_{10}$ reduction</td>
<td>1.80</td>
<td>0.96</td>
<td>0.75*</td>
<td>0.58*</td>
</tr>
<tr>
<td>(µg/mL)/MIC for -4 log$_{10}$ reduction</td>
<td>-1.27</td>
<td>0.91*</td>
<td>0.87*</td>
<td></td>
</tr>
</tbody>
</table>

$E_0$ is the difference in bacterial count between 0 and 24 h (log$_{10}$ cfu/mL); $E_{max}$ is the difference in log$_{10}$ cfu/mL in samples incubated with combined concentrations of EFX and CFX between time 0 and 24 h when the detection limit (10 cfu/mL) is reached; Efficacy is expressed as log$_{10}$ reduction of initial bacterial count; $EC_{50}$ is the activity of antibiotics producing 50% of the $E_{max}$; $γ$ is the Hill coefficient which describes the steepness of the antibacterial effect.

Table 2. Descriptive pharmacodynamic parameters estimated by fitting of the relationship of the bacterial count (log$_{10}$ cfu/mL) after 24 h incubation and the initial inoculums bacterial count of E. coli ATCC 25922. The multiples of MIC of EFX, CFX and the sum of the multiples of MIC of both antibiotics necessary to achieve bacteriostatic effect, bactericidal effect and virtual bacterial eradication are presented.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EFX</th>
<th>CFX</th>
<th>(EFX+CFX)</th>
<th>(EFX+CFX)BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC$_{e}$ (µg/mL)</td>
<td>0.0278</td>
<td>0.0103</td>
<td>0.0070</td>
<td>0.0049</td>
</tr>
<tr>
<td>MBC$_{e}$ (µg/mL)</td>
<td>0.0562</td>
<td>0.0150</td>
<td>0.0098</td>
<td>0.0068</td>
</tr>
<tr>
<td>MBE$_{e}$ (µg/mL)</td>
<td>NC</td>
<td>0.0198</td>
<td>0.0118</td>
<td>0.0083</td>
</tr>
</tbody>
</table>

MIC$_{e}$, MBC$_{e}$ and MBE$_{e}$ are the values of minimum inhibitory concentration, minimum bactericidal concentration and minimum eradication concentration respectively which were estimated by fitting of the experimental data by non-linear regression analysis using the Hill model respectively. Other symbols were previously explained.

Table 2. Descriptive pharmacodynamic parameters estimated by fitting of the relationship of the bacterial count (log$_{10}$ cfu per milliliter) for E. coli ATCC 25922 versus concentrations of (A) EFX, (B) CFX, (C) (EFX+CFX), and (D) (EFX+CFX)BS by non-linear regression analysis using the Hill model.
ANTIBACTERIAL ACTIVITY OF EFX-CFX AND EFX-CFX-BS

The evolution of the viable cell count as a function of several concentrations of (EFX+CFX) and (EFX+CFX)BS are presented in Figure 1C and D respectively.

The visual inspection of the figures showed that a bactericidal activity was only attained by (EFX+CFX) at a concentration equivalent to 0.84 x MIC. On other hand, all concentrations of (EFX+CFX) and (EFX+CFX)BS in the range of 1.68 x MIC to 53.85 x MIC attained bacterial eradication.

Plots of the relationship between (EFX+CFX) and (EFX+CFX)BS and log<sub>10</sub> difference between bacterial count (cfu/mL) after 24 h incubation and the initial inoculums bacterial count are present in Figure 2 C and D respectively. Pharmacodynamic parameters resulting from fitting of the experimental data with the sigmoid E<sub>max</sub> model are presented in Table 1.

Both antibiotics combinations presented slope values higher than 1, which indicated an all-or-nothing concentration-effect. On other hand, BS increased the potency of (EFX+CFX), so the estimated EC<sub>50</sub> value was low in the presence of BS. As consequence, bacteriostatic, bactericidal and bacterial eradication effects of (EFX+CFX) BS were attained at lower multiples of MIC than those obtained by (EFX+CFX). Moreover negligible differences in terms of efficacy were observed at concentrations higher than 1 x MIC for (EFX+CFX) and 1.68 x MIC for (EFX+CFX)BS (Table 1).

MIC VALUES OF EFX AND CFX DETERMINED IN SURVIVING BACTERIA

The surviving bacteria after 24 h exposure to EFX, CFX and EFX+CFX presented identical MIC values as the parent culture from which they were derived, being 0.0312 and 0.0156 µg/mL for EFX and CFX respectively.

BACTERIAL GROWTH KINETICS

The bacterial growth rate constant in absence of antibiotics presented values in the range of 1.42 h<sup>-1</sup> to 1.53 h<sup>-1</sup>, being the values of t<sub>1/2g</sub> comprised within 0.48 to 0.45 h. On other hand in the presence of BS the estimated growth rate decreased to 0.98 h<sup>-1</sup> which resulted in a t<sub>1/2g</sub> value of 0.71 h.

BACTERIAL ELIMINATION KINETICS RESULTED BY EFFECT OF EFX, CFX, (EFX+CFX) AND (EFX+CFX)BS

The evolution of viable bacterial count of E. coli (ATCC 25922) versus time after 24 h exposure to concentrations greater than 1 x MIC for EFX and CFX, and than 1.68 x MIC for (EFX+CFX) and (EFX+CFX)BS presented similar bacterial elimination kinetic profiles and efficacy (Figure 1).

In general terms, they presented a biphasic killing pattern, in which the initial phase was a rapid killing of the bulk population, and the second phase was a slow bacterial of a small bacterial sub-population. Some of these profiles were fitted with a two exponential model and some were fitted by a three exponential model as presented in Figure 3A, B, C and D.

Both antibiotics combinations presented slope values higher than 1, which indicated an all-or-nothing concentration-effect. On other hand, BS increased the potency of (EFX+CFX), so the estimated EC<sub>50</sub> value was low in the presence of BS. As consequence, bacteriostatic, bactericidal and bacterial eradication effects of (EFX+CFX) BS were attained at lower multiples of MIC than those obtained by (EFX+CFX). Moreover negligible differences in terms of efficacy were observed at concentrations higher than 1 x MIC for (EFX+CFX) and 1.68 x MIC for (EFX+CFX)BS (Table 1).
The first and the second terms of the model were interpreted as a mathematical expression of the rapid bacterial elimination kinetics of antibiotic-susceptible bacterial sub-populations. On other hand, the third exponential term was associated to the slow elimination kinetics of the persister bacterial sub-population, and its coefficient \( (N_z) \) was interpreted as a estimated bacterial sub-population of persister cells expressed as percent of the initial bacterial count. The pharmacodynamic parameters calculated by fitting of the kill-curves profiles are presented in Table 3.

**Discussion**

The potency and the efficacy of CFX were higher than EFX, which was evidenced by the low values of EC\(_{50}\) and the high values of E\(_{max}\) observed for CFX than EFX.

The study of the relationship between the concentrations and efficacy of EFX and CFX evidenced an all-or-nothing response. This means that small increases in concentrations of EFX and CFX determined that their efficacies changed from a mere reduction of bacterial growth to an efficacy equivalent to a bactericidal (EFX) or bacterial eradication (CFX) effect. Our result shown that concentrations above 1 x MIC of EFX and CFX reached an antibacterial efficacy equivalent to bactericidal activity (-3 log\(_{10}\) \( N_0 \)) and bacterial eradication (-4 log\(_{10}\) \( N_0 \)) respectively.

This all-or-nothing pharmacodynamic behavior, seems to be characteristic of FQs agents, because some authors have reported that the FQs drugs reached their maximum efficacy with concentration between 1 x MIC to 4 x MIC, for example: EFX against *E. coli* [6, 15, 28], norfloxacin against *Staphylococcus aureus* [14], marbofloxacin against *E. coli* [13], sarafloxacin against *E. coli* [35].

An interesting finding was that all supra-inhibitory concentrations of EFX and CFX lead similar bacterial elimination profiles (Figure 1), in which the concentration-dependent activity of EFX and CFX was observed during the first 5 h of exposure, and from this time all concentrations above 1 x MIC lead similar slow bacterial elimination and efficacy.

This finding could be explained by the selection of a persister sub-population originated because of 24 h exposure to supra-inhibitory concentrations of EFX and CFX. This prolonged antibiotic exposure gave rise to regular cells died, while persister cells survived.

The slow elimination of persister cells creates the impression of an apparent time-concentration mode of action for EFX and CFX. Moreover EFX and CFX did not change their mode of action on persister cells, but their antibacterial activity was reduced. Many authors attribute the tolerance of persister cells to antibiotics due a state in which their metabolic state is reduced and the growth arrested. In this sense, the tolerance to antibiotics is a consequence of an attenuated cell respiration [21], thus this state of dormancy should be the responsible of preclude the activity of antibiotics. Moreover a growing number of evidences indicate that the link between the metabolic state of bacterial populations and their phenotype of susceptibility to antibiotics is far more complex [9].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>EFX</th>
<th>CFX</th>
<th>(EFX+CFX)</th>
<th>(EFX+CFX)BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N_1 ) (%)</td>
<td>98,8</td>
<td>99,7</td>
<td>87,6</td>
<td>99,2</td>
</tr>
<tr>
<td>( k_{b1} ) (h(^{-1}))</td>
<td>5,34</td>
<td>5,21</td>
<td>6,11</td>
<td>5,34</td>
</tr>
<tr>
<td>( t_{1/2b1} ) (h)</td>
<td>0,219</td>
<td>0,164</td>
<td>0,154</td>
<td>0,163</td>
</tr>
<tr>
<td>( N_2 ) (%)</td>
<td>4,14</td>
<td>3,48</td>
<td>13,05</td>
<td>2,42</td>
</tr>
<tr>
<td>( k_{b2} ) (h(^{-1}))</td>
<td>0,640</td>
<td>0,769</td>
<td>1,30</td>
<td>0,525</td>
</tr>
<tr>
<td>( t_{1/2b2} ) (h)</td>
<td>1,10</td>
<td>0,943</td>
<td>0,598</td>
<td>1,36</td>
</tr>
<tr>
<td>( N_3 ) (%)</td>
<td>0,434</td>
<td>0,207</td>
<td>0,426</td>
<td>0,835</td>
</tr>
<tr>
<td>( k_{b3} ) (h(^{-1}))</td>
<td>0,131</td>
<td>0,156</td>
<td>0,208</td>
<td>0,199</td>
</tr>
<tr>
<td>( t_{1/2b3} ) (h)</td>
<td>5,56</td>
<td>4,55</td>
<td>3,43</td>
<td>3,64</td>
</tr>
</tbody>
</table>

\( N_1, N_2, N_3 \) (%) are estimated bacterial sub-populations expressed as percent of the initial bacterial count; \( k_{b1}, k_{b2}, k_{b3} \) (h\(^{-1}\)) are theoretical first order bacterial kill rate constant of each exponential term; \( t_{1/2b1}, t_{1/2b2}, t_{1/2b3} \) (h) are the estimated half-life of bacterial elimination of each estimated bacterial sub-population. Other symbols were explained previously. Mean and SD of \( N_1, k_{b1}, t_{1/2b1} \) and \( N_2, k_{b2}, t_{1/2b2} \) were estimated by \( n = 4 \) for EFX and CFX and \( n = 6 \) for (EFX+CFX) and (EFX+CFX)BS. Mean and SD of \( N_3, k_{b3}, t_{1/2b3} \) were estimated by \( n = 2 \) for EFX and CFX and \( n = 4 \) for (EFX+CFX) and (EFX+CFX)BS.

Table III: Pharmacodynamic parameters estimated fitting of time-kill curves of *E. coli* ATCC 25922 exposed to (A) EFX, (B) CFX, (C) (EFX+CFX), and (D) (EFX+CFX)BS at concentrations equivalent to multiples of MIC >1. The parameters were estimated by fitting of the experimental data by non-linear regression analysis using a multi exponential model.

Although persister formation has been attributed to cell dormancy, these cells showed less cytoplasmic drug accumulation as a result of enhanced efflux activity. Consistently a number of multi-drug efflux genes, particularly the central component TolC, show higher expression in persisters [27].

Our results showed that the growth of E. coli ATCC 25922 was negatively affected by the presence of BS. Accordingly growth rate was low in the presence of BS than in the standard culture medium, which could be associated with the antibacterial activity of BS components.

The visual inspection of time-kill curves presented in figures 1 and 2 showed that (EFX+CFX) presented high efficacy than the observed for CFX. Notwithstanding the antibacterial activity of SB, no additional efficacy was obtained in its presence, but increased the potency of (EFX+CFX).

In concordance to the observed for EFX and CFX, similar bacterial kinetic elimination profiles were observed for (EFX+CFX) and (EFX+CFX)BS at concentrations above 1 x MIC, and after 5 h exposition to these concentrations and only a selection of a sub-populations of persister cells were attained. On other hand, even in the presence of BS, a 24 h exposition of E. coli to supra-inhibitory concentrations of (EFX+CFX) and (EFX+CFX)BS was not enough to eradicate persister cells.

Although this phenomenon was first reported in the last century [3], the clinical impact is that in the absence of appropriate host immunity, persisters that survive antibiotic treatment are more likely to thrive. As consequence of this, recurrent or intractable infections arise. On other hand persister cells behave as an evolutionary reservoir from which resistant bacteria can emerge [8].

The visual inspection of semilogarithmic plot of the time-kill curves of E. coli ATCC 25922 showed that bacterial elimination profiles produced by all concentration above 1 x MIC of EFX, CFX, and above 1,68 x MIC o (EFX+CFX) and (EFX+CFX)BS presented at least two well defined elimination phases. By the way, some bacterial kinetic elimination profiles were fitted by a two exponential model and some were fitted by a three exponential model. This finding showed that the whole bacterial population of E. coli ATCC 25922 was not homogeneous and different antibiotic sensitivity bacterial sub-populations were present. A cause of this, each exponential term was interpreted as a descriptive mathematical expression of kinetic elimination of a bacteria sub-population. The third exponential term was related with the slow disappearance of persister cells sub-population.

It is worldwide accepted the concentration-dependent mode of action of FQs. Moreover in this study the selection of a sub-population of persister cells was a direct consequence of the antibacterial activity of EFX and CFX.

It is commonly accepted that persister cells are a small bacterial sub-population present in an initial inoculum before any antibiotic treatment which spontaneously enter a dormant, non-dividing state [20]. A cause of this, the exposure to supra-inhibitory concentrations of EFX and CFX exerts pressure selecting persisters. This explains the reason why even though FQs antimicrobials are potent bactericidal antibiotics they cannot sterilize a bacterial [12].

Moreover it was demonstrated that FQs antimicrobials induce the persister formation mediated by a SOS response, challenging the prevailing view that persisters are pre-existing and formed purely by stochastic means [33].

In consequence, a biphasic pattern of bacterial elimination kinetic (fast elimination of the sensible sub-population and slow elimination of persisters cells) are two simultaneous consequences of the antibacterial activity of EFX and CFX that cannot be evaluated separately. On the contrary, they constitute two ways of the same antibacterial activity.

This hypothesis is reinforced by the fact that all efficacies of bacterial kinetic elimination profiles obtained by concentrations above 1 x MIC of EFX, CFX, and 1.68 x MIC of (EFX-CFX) and (EFX-CFX)BS were similar.

Notwithstanding our findings, high concentrations of EFX and CFX are necessary to avoid the emergence of resistant strains [4].

Nevertheless from the results obtained in this study, two questions arise: Are CFX and EFX antibiotics whose mode of action should still be exclusively considered concentration-dependent agents? Should the time of exposition be incorporated into the PK-PD index for predicting their efficacies?

This topic was considered by Blot et al., (2014), who classified FQs agents in an intermediate level between antibiotics which exhibit time-dependent and concentration-dependent mode of action, considering this kind of antibacterial agents as concentration-dependent with time dependence mode of action [5].

Conclusion

As conclusion from the results obtained in this study, it is possible to infer that the selection of persister cells sub-population of E. coli ATCC 25922 is a consequence of the antibacterial activity of EFX and its metabolite CFX, and its elimination depended on the high concentration and the time of exposure to both drugs.

These kinds of cells represent a new target for discovery of drugs capable of minimizing the emergence of them, and leading to the effective treatment of current and chronic infections.
The in vivo relevance of these in vitro results merits further investigation in animal models of infection to ensure the proper use of EFX.

Acknowledgments

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References


