Research in Veterinary Science 98 (2015) 98–105

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

In vitro evaluation of synergistic activity between ciprofloxacin and broad snouted caiman serum against *Escherichia coli*

P.A. Siroski [a,b,*], N.B. Russi [c], H.H. Ortega [a], E.A. Formentini [d]

[a] *ICIVET-CONICET-Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, R.P. Kreder 2805, CP S3080HOF Esperanza, Santa Fe, Argentina*
[b] *Laboratorio de Zoología Aplicada: Anexo Vertebrados (FHUC-UNL/MASPyMA) Departamento de ciencias Naturales, Facultad de Humanidades y Ciencias, Universidad Nacional del Litoral, Ciudad Universitaria -Paraje El Pozo, CP S3000 Santa Fe, Argentina*
[c] *Laboratorio de Bacteriología del Hospital de Salud Animal-Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, R.P. Kreder 2805, CP S3080HOF Esperanza, Santa Fe, Argentina*
[d] *Cátedra de Farmacología-Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, R.P. Kreder 2805, CP S3080HOF Esperanza, Santa Fe, Argentina*

ARTICLE INFO

Article history:
Received 27 February 2014
Accepted 6 November 2014

Keywords:
Ciprofloxacin
Escherichia coli
Caiman serum
Synergism
Zootechnical interest

ABSTRACT

The in vitro synergistic activity between ciprofloxacin and serum of broad snouted caiman on *Escherichia coli* was studied. The estimated MIC value of ciprofloxacin was 0.0188 μg/ml, and two assays of kill curve during 5 hours were performed: the first one in a standard culture medium and the second one in the presence of caiman serum. Different concentrations of ciprofloxacin were tested. Ciprofloxacin showed higher values of bacterial elimination rate in the presence of caiman serum in all concentrations tested. The combined activity of sub-inhibitory concentrations of ciprofloxacin and the humoral immune factors present in caiman serum determined an increase in the bacterial elimination observed in this assay. We suggest that the antibacterial activity of complement and natural antibodies present in caiman serum, which can bind to both Gram-negative and Gram-positive bacteria and acting through the classical complement pathway, can inhibit bacterial growth of *Escherichia coli* by lysis.

1. Introduction

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 (Mueller et al., 2004). In the last three decades, certain in vitro properties of these agents as post-antibiotic effect (PAE) and the antibacterial activity of sub-inhibitory concentrations (sub-MIC effect) (Odenholt, 2001) 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 antibiotic concentrations versus time (Liu et al., 2002).

However, the in vitro interaction between antibiotic and bacteria is different from that in a living organism, due to the lack of antibacterial activity from components of the host immune system (De Leo et al., 2009). Accordingly, the in vitro studies are conducted under conditions that resemble those found in an immunocompromised patient (White, 2001). Currently, the evaluation of the in vitro activity of antibiotics intended to simulate conditions present in the in vivo scenario, in order to evaluate the interaction between the antibiotic, the bacteria and the living organism (immune response), so that this information can be used for the design and optimization of more effective dosing schedules.

Mostly, the antibiotic therapy is not able to eliminate completely a microbial infection, but it usually has the collaboration of the immune system to work together with the same objective. Normally, the correlation between an in vitro susceptibility result and clinical outcome is never 100%. Cases treated with a seemingly appropriate antimicrobial (i.e. one to which the microorganism is susceptible in vitro) may still fail to respond clinically, while those treated with an inappropriate antibiotic (i.e. one to which the microorganism is resistant) may still show a favorable clinical response. This is because other factors than antibiotics play a role in determining outcomes (Blondeau, 2009).

Clinical experience indicates that the success of antimicrobial therapy depends on the normal function of the defense system of the patient (Kristian et al., 2007; Mehrzad et al., 2009), which is necessary for successful recovery from infectious diseases (Blondeau, 2009), so it is important to realize that the organisms must cure himself, since antibiotic treatment only helps indirectly by combating the microorganism (Blondeau, 2009; Mattie, 1994).

However, the intensive and inappropriate use of antibiotics and adaptive capacity of bacterial genome have caused a world-wide

* Corresponding author. ICIVET-CONICET-Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, R.P. Kreder 2805, CP S3080HOF-Esperanza, Santa Fe, Argentina. Tel.: +54 342 4579256; fax: +54 342 4579256.
E-mail address: psiroski@fcv.unl.edu.ar (P.A. Siroski).

http://dx.doi.org/10.1016/j.rvsc.2014.11.007
0034-5288/

emergent phenomenon of antibiotic resistance, and a growing number of bacterial species are becoming resistant to more than one antibiotic classes, creating multidrug-resistant (MDR) bacterial strains (Foster, 2004; Piras et al., 2012). For this reason, research is directed toward the understanding of how the immune system may be manipulated for controlling some bacterial diseases (Sparo and Sanchez Bruni, 2012).

Immunity of animal species has evolved from innate mechanisms to more complex and adaptable immune responses designed to recognize materials unknown for them. The concept of innate immunity refers to the first line host defense that serves to restrain infection in the early hours after exposure to microorganism (Hoffmann et al., 1999).

Crocodilians, like much more wild animals, exhibit strongly marked social behaviors and responses to stressors that can trigger serious disputes between co-specifics, predators and, even, conflict with human activities. As a result they could show trauma, serious injuries and the loss of members. Generally, these animals live in environments containing a high concentration of pathogenic microorganisms in both natural and captive systems. In many cases, the combination of these factors could cause a local or systemic infectious process, however, crocodilians tolerate these situations and generally, without showing signs of illness (Siroski et al., 2009). This phenomenon can be explained because blood contains important elements that mediate rapid responses to infection (Levy, 2000), and acute and recurrent inflammatory processes originate high levels of acute phase proteins including complement proteins (C3) which are involved in bacterial opsonization and lysis (Piras et al., 2014). Accordingly, antimicrobial activity of crocodilian blood was described in some species (Britton et al., 2002; Merchant et al., 2003; (Siroski et al., 2009; 2010), and plasma of broad snouted caiman (*Caiman latirostris)* demonstrated a time-dependent bactericidal activity against *E. coli* (Siroski et al., 2009).

Ciprofloxacin is an antibacterial agent which exhibits concentration-dependent effect, this in the higher concentration is the higher kill-rate (Kays and Denys, 2001), and it has shown to have activity against *E. coli*, which is the etiologic agent of gastrointestinal infections and sepsis in humans and animals, and respiratory tract infections in animals (Katie et al., 2005).

Up to our knowledge, no information is available about the combined antibacterial activity such from antibiotics and natural components present in caiman serum with antibacterial activities without treatment. So the objective of this study was to investigate and quantify the in vitro bactericidal activity of ciprofloxacin against *E. coli* (ATCC 25922) in the presence of broad snouted caiman serum (CS).

2. Materials and methods

2.1. Microorganism and antibiotic

A strain of *E. coli* (ATCC 25922) and ciprofloxacin (Sigma-Aldrich, Chemical Company, St. Louis, USA) was selected to perform this assay.

2.2. Broad-snouted caiman (Caiman latirostris) serum

Blood samples from adult caimans ($n = 4$) (Proyecto Yacaré, Convenio Gobierno de Santa Fe/MUPCN, Santa Fe Province, Argentina) were taken from the spinal vein (Tourn et al., 1994; Zippel et al., 2003). Blood was allowed to clot and then centrifuged at $2500 \times g$ for 15 min. Then CS was removed and maintained at –20 °C for subsequent analysis.

2.3. 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 (1×10^8 colony forming unit [CFU]/ml) measured with a turbidimeter. After that, serial dilutions were performed to obtain a final concentration of 1×10^6 CFU/ml.

2.4. Determination of MIC values

The MIC of ciprofloxacin on the stain of *E. coli* (ATCC 25922) was determined by macrodilution method (CLSI, Clinical and Laboratory Standards Institute, 2008) in Müller–Hinton broth (MHB) (Britania, Buenos Aires, Argentina). The growth medium was prepared according to the manufacturer's instructions and autoclaved prior to use at 121 °C (15 min/l). Broth was prepared the day before of the assay, so that it was stored in a refrigerator at approximately 7 °C until use. The MIC of ciprofloxacin on *E. coli* was determined in the range of concentrations of 0.00236–1.2032 μg/ml.

2.5. Bacterial growth curves

Bacterial cultures in MHB and MHB/CS 50:50 adjusted to 1×10^6 CFU/ml were incubated at 35 °C during 5 h. Aliquots (100 μl) were removed from the incubation cultures at 0, 0.5, 1, 2, 3, 4 and 5 h and then were serially diluted in sterile isotonic saline solution. Aliquots (100 μl) of the final dilutions were spread in duplicate on to agar plates, and the colonies were counted after overnight incubation at 35 °C.

2.6. Time–kill curves

Bacterial cultures in MHB and MHB/CS 50:50 adjusted to 1×10^6 CFU/ml were exposed to constant concentrations of ciprofloxacin ($0.25 \times$ MIC, $0.5 \times$ MIC, $1 \times$ MIC, $4 \times$ MIC, $8 \times$ MIC, $16 \times$ MIC and $32 \times$ MIC) and then incubated at 35 °C during 5 h. Aliquots (100 μl) were removed from the incubation cultures at 0, 0.5, 1, 2, 3, 4 and 5 h and then were serially diluted in sterile isotonic saline solution. Aliquots (100 μl) of the final dilutions were spread in duplicate on to agar plates, and the colonies were counted after overnight incubation at 35 °C.

2.7. 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 death and growth curve, the number of viable bacteria was expressed as the average of counts made in each replicate ($n = 2$). In order to facilitate the construction and interpretation of the growth and death bacterial curves, the values of viable bacterial count were expressed as $\log_{10}$ CFU/ml.

2.8. 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 MHB and MHB-CS were fitted with Equation 1, which considers the limited available nutrients and space of the in vitro system.

$$\frac{dN}{dt} = k_g \cdot \left(1 - e^{-zt}\right) \cdot N \quad (1)$$

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 of antibiotics, z is an adaptation rate constant for describing a delayed effect in growth and N (CFU/ml) is the number of viable bacteria. The data obtained in the time–kill curves of *E. coli* (ATCC 29213) in the presence of constant concentrations of ciprofloxacin in MHB and MHB-CS were fitted with Equation 2.

$$\frac{dN}{dt} = \left[\left(k_g - k_{elb}\right) \cdot \left(1 - e^{-zt^a}\right)\right] \cdot N \quad (2)$$

where k_g (h^{-1}) is the bacterial growth rate constant estimated from the fitting of the growth curves in MHB and MHB-CS respectively, and k_{elb} (h^{-1}) is the bacterial kill rate constant resulting from the activity of ciprofloxacin in MHB and the activity of ciprofloxacin and intrinsic bactericidal activity of CS and (a) is a constant used to fit the phenotypic tolerance phenomenon. Other symbols were explained previously. In order to evaluate the effect of the caiman serum on the bacterial elimination rate of E. coli, the time required for the bacterial viable count to reduce by 50% (t_r) at several concentrations in MHB and MHB-CS was estimated as: $\ln 2/k_{elb}$. The relationship between ciprofloxacin concentrations and the estimated bacterial elimination rate (k_{elb}) in MHB was fitted with the commonly used Emax model:

$$k_{elb} = \frac{k_{max} \cdot C^n}{C_{50}{}^n + C^n} \quad (3)$$

where k_{max}, (h^{-1}) is the maximum bacterial elimination rate, C (μg/ml) is the fixed ciprofloxacin concentration, C_{50} (μg/ml) is the concentration of ciprofloxacin necessary to produce 50% of the maximum effect and n is the Hill coefficient or shape factor. Other symbols were explained previously. The relationship between ciprofloxacin concentrations and the estimated bacterial elimination rate (k_{elb}) in MHB-CS was fitted using a linear model:

$$k_{elb} = b + (a \cdot C) \quad (4)$$

where a is the slope of the line; b is the y-intercept of the line. Other symbols were explained previously.

2.9. Quantification of time–kill curves and antimicrobial effect

The activity of ciprofloxacin in MHB and MHB-CS on *E. coli* (ATCC 25922) was quantified by different parameters:

2.9.1. Descriptive parameters

N_0 is the initial viable bacterial count, N_{tn} is the number of bacteria resulting from the exposure to antibiotic at the end of the experiment, t_0 and t_n are the start time and the end time respectively. In this study the kill measurement was determined by the actual reduction in viable counts at t_n (5 h).

2.9.2. Integral parameters

Some integral parameters reported by (Firsov et al., 1997) were used to quantitate viable counts; AUBC is the area under the viable bacterial count exposed to antibiotic from t_0 to t_n; AAC is the area above the N curve and under the baseline around the N_0 level from t_0 to t_n. AAC was calculated as $AUCN_0 - AUBC$, being $AUCN_0$ the algebraic sum of the areas around the N_0 level and represent an equilibrium of the bacteria growth and death over time (Fig. 1). The areas under the curves were calculated by the linear trapezoidal rule method reported by Baggot (2001).

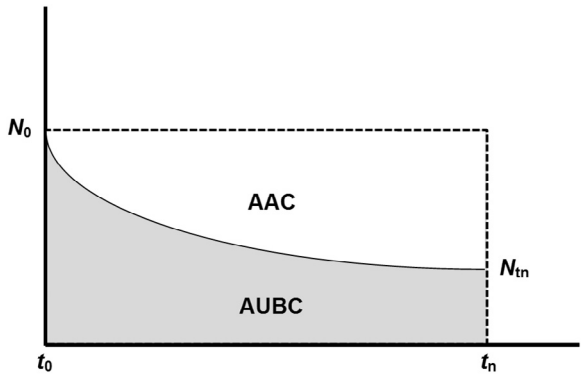

Fig. 1. Parameters for quantitating bacterial killing curve and the antibacterial effect; N_0 is the initial inoculum, N_{tn} is the number of bacteria resulting from the exposure to antibiotic at the end of the experiment, t_0 and t_n are the start time and the end time respectively, AAC is the algebraic sum of the areas around N_0 level from t_0 to t_n, AUBC is the area under the viable bacterial count exposed to antibiotic from t_0 to t_n.

2.8.3. Efficacy parameters

The bactericidal effect was defined as a 3log decrease in the N_0 or a 99.9% kill over at t_n (NCCLS, National Committee for Clinical Laboratory Standards, 1992). The percent of reduction of bacteria biomass exposed at constant concentrations of ciprofloxacin during 5 h in MHB and MHB-CS was estimated using the integral endpoints of the antimicrobial effect by the following equation:

$$\text{Reduction of bacteria biomass \%} = (AAC/AUBN_0) \times 100$$

3. Results

The estimated MIC value of ciprofloxacin against *E. coli* (ATCC 25922) was 0.0188 μg/ml, and the time evolution of viable count in function of several concentrations of ciprofloxacin in MHB and MHB-CS are presented in Fig. 2. The activity of ciprofloxacin in the

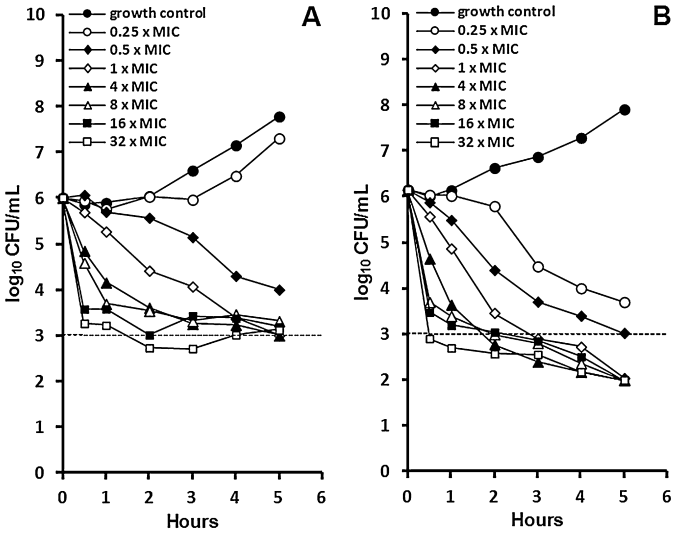

Fig. 2. Time–kill pharmacodynamics curves of *E. coli* (ATCC 25922) in (A) Müller–Hinton broth (MHB) and (B) Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS) in the absence of antibiotic (control) or in the presence of constant concentrations of ciprofloxacin; 0.0047 μg/ml (0.25 × MIC), 0.0094 μg/ml (0.5 × MIC), 0.0188 μg/ml (1 × MIC), 0.0752 μg/ml (4 × MIC), 0.1504 μg/ml (8 × MIC), 0.3008 μg/ml (16 × MIC) and 0.6016 μg/ml (32 × MIC). The dotted line represents the baseline that indicates the reduction of the initial inoculum 1000-fold (99.9%). Each datum point represents the mean CFU per milliliter for duplicate experiments.

Table 1
Viable bacterial count expressed as $\log_{10}$ CFU/ml of *E. coli* (ATCC 25922) in the presence of constant concentrations of ciprofloxacin during 5 h in Müller–Hinton broth (MHB). The reduction of the initial inoculum 1000-fold (99.9%) are in bold.

Time (hours)	Growth control	Ciprofloxacin (μg/ml)						
		0.0047	0.0094	0.0188	0.0752	0.1504	0.3008	0.6016
		0.25 × MIC	0.5 × MIC	1 × MIC	4 × MIC	8 × MIC	16 × MIC	32 × MIC
0	6.01	6.01	6.01	6.01	6.01	6.01	6.01	6.01
0.5	5.86	5.94	6.06	5.69	4.85	4.59	3.58	3.26
1	5.89	5.76	5.70	5.27	4.15	3.69	3.58	3.22
2	6.04	6.03	5.57	4.42	3.61	3.54	**3.01**	**2.73**
3	6.60	5.96	5.15	4.07	3.25	3.31	3.43	**2.72**
4	7.15	6.48	4.30	3.38	3.23	3.46	3.37	3.02
5	7.78	7.30	4.00	**3.00**	**3.00**	3.31	3.19	3.12

Table 2
Viable bacterial count expressed as $\log_{10}$ CFU/ml of *E. coli* (ATCC 25922) in the presence of constant concentrations of ciprofloxacin during 5 h in Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS). The reduction of the initial inoculum 1000-fold (99.9%) are in bold.

Time (hours)	Growth control	Ciprofloxacin (μg/ml)						
		0.0047	0.0094	0.0188	0.0752	0.1504	0.3008	0.6016
		0.25 × MIC	0.5 × MIC	1 × MIC	4 × MIC	8 × MIC	16 × MIC	32 × MIC
0	6.15	6.15	6.15	6.15	6.15	6.15	6.15	6.15
0.5	6.00	6.04	5.88	5.57	4.65	3.70	3.48	**2.90**
1	6.14	6.02	5.49	4.87	3.64	3.40	3.19	**2.70**
2	6.62	5.79	4.40	3.46	**2.78**	**3.00**	**3.04**	**2.57**
3	6.87	4.48	3.71	**2.90**	**2.40**	**2.81**	**2.85**	**2.56**
4	7.28	4.00	3.40	**2.72**	**2.18**	**2.36**	**2.51**	**2.18**
5	7.90	3.70	**3.03**	**2.04**	**2.00**	**2.00**	**2.00**	**2.00**

presence of CS was higher than standard culture medium ($p < 0.05$). Bacterial growth was observed at concentration of 0.0047 μg/ml (MIC × 0.25) of ciprofloxacin in MHB (Fig. 2A), whereas a decrease in viable counts was shown in MHB-CS at the same antibiotic concentration (Fig. 2B).

In general, an efficacy of 99.9% at t_n and a faster decrease of viable count at ciprofloxacin concentrations equivalent to 0.5 × MIC, 1 × MIC, 4 × MIC, 8 × MIC, 16 × MIC and 32 × MIC were detected in MHB-CS than in MHB (Tables 1 and 2).

Reduction of bacterial biomass was higher at the range of concentrations of 0.25 × MIC and 1 × MIC in the presence of CS than in MHB (Fig. 3 and Table 3).

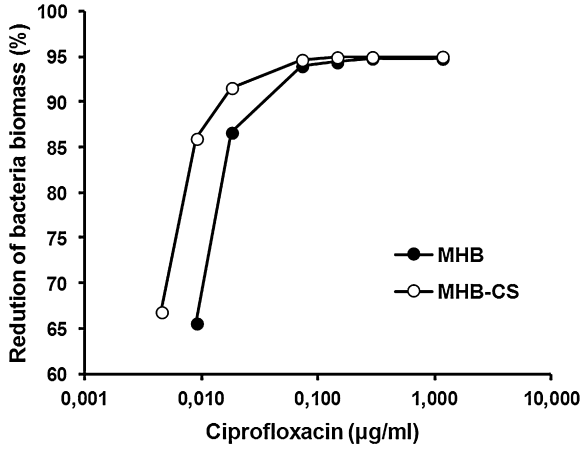

Fig. 3. Reduction of the bacterial biomass expressed as percent of viable bacteria eliminated of a strain of *E. coli* (ATCC 25922) in the presence of constant concentrations of ciprofloxacin; 0.0047 μg/ml, 0.0094 μg/ml, 0.0188 μg/ml, 0.0752 μg/ml, 0.1504 μg/ml, 0.3008 μg/ml and 0.6016 μg/ml, during 5 h in Müller–Hinton broth (MHB) and in Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS).

The fitted curves of ciprofloxacin against the bacterial strain in MHB and MHB-CS are shown in Fig. 4, and pharmacodynamics parameters for each antibiotic concentration are listed in Table 4. The results show that the model chosen was appropriated for fitting the data.

Summarizing the data, ciprofloxacin showed high values for the estimated bacterial elimination rate (k_{elb}) in the presence of CS in all tested concentrations, however these differences were notoriously higher at low concentrations of 0.25 × MIC to 1 × MIC (Table 4).

The fitting of the dose–response relationship between ciprofloxacin concentrations and k_{elb} in MHB and MHB-CS are depicted in Fig. 5, and the estimated pharmacodynamic parameters are presented in Table 5.

At identical levels of ciprofloxacin, the values of k_{elb} obtained in the presence of CS were greater than in the standard culture medium. It was also noted a linear relationship between concentrations of ciprofloxacin and values of k_{elb} in MHB-CS; whereas in MHB, these

Table 3
Reduction of bacteria biomass of an inoculum of *E. coli* (ATCC 25922) exposed at constant concentrations of ciprofloxacin during 5 h in Müller–Hinton broth (MHB) and Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS). The percent of reduction was estimated using the integral endpoint of the antimicrobial effect.

Reduction of bacteria biomass (%)			
Ciprofloxacin (μg/ml)	Culture medium		*diff.*
	MHB	MHB-CS	
0.0047 (0.25 × MIC)	–	66.81	66.8
0.0094 (0.5 × MIC)	65.57	85.93	20.4
0.0188 (1 × MIC)	86.60	91.53	4.93
0.0752 (4 × MIC)	93.94	94.62	0.69
0.1504 (8 × MIC)	94.36	94.91	0.95
0.3008 (16 × MIC)	94.77	94.93	0.16
0.6016 (32 × MIC)	94.75	94.98	0.22

diff is the difference between MHB-CS values and MHB values.

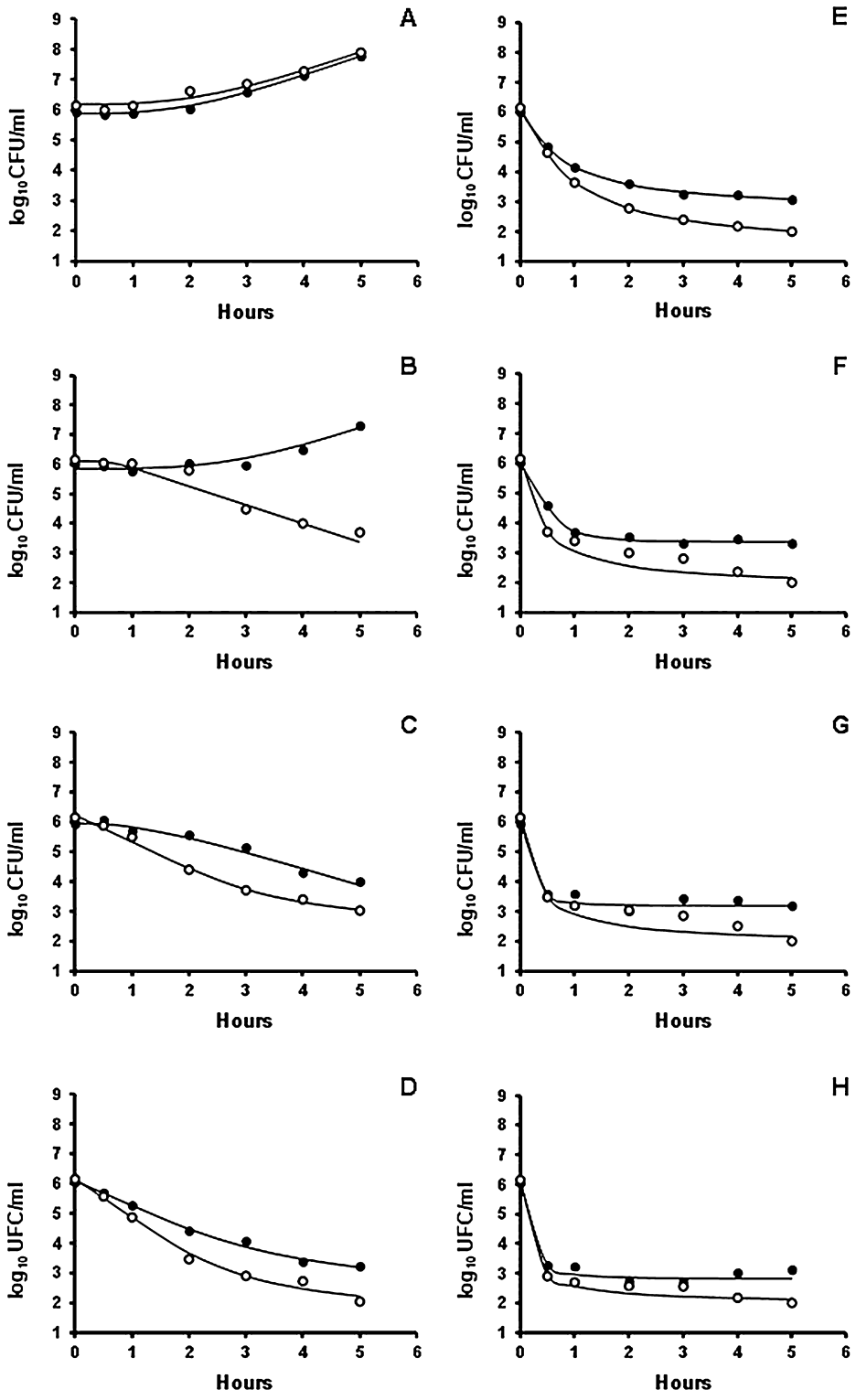

Fig. 4. Fitted curves of *E. coli* (ATCC 25922) in (●) Müller–Hinton broth (MHB) and (○) Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS) in the absence of antibiotic (A) or in the presence of constant concentrations of ciprofloxacin; 0.0047 μg/ml (B), 0.0094 μg/ml (C), 0.0188 μg/ml (D), 0.0752 μg/ml (E), 0.1504 μg/ml (F), 0.3008 μg/ml (G) and 0.6016 μg/ml (H). Each datum point represents the mean CFU per milliliter for duplicate experiments.

values showed a tendency to diminish progressively at high concentrations of the antibiotic. As consequence, the antibacterial activities of several concentrations of ciprofloxacin against *E. coli* (ATCC 25922) expressed as t_r were lower in the presence of MHB-CS than the observed in MHB (Table 6).

4. Discussion and conclusion

Dissemination of resistance is given by inappropriate use of antibiotics by clinicians in human and veterinary medicine and agricultural community (Wright, 2010). The contribution to the

Table 4
Pharmacodynamic parameters of ciprofloxacin against *E. coli* (ATCC 25922) in Müller–Hinton broth (MHB) and Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS).

Ciprofloxacin (μg/ml)		MHB				MHB-CS			
		k_g (h^{-1})	k_{elb} (h^{-1})	z	a	k_g (h^{-1})	k_{elb} (h^{-1})	z	a
0.0000	Control	1.512	–	–	–	1.472	–	–	–
0.0047	0.25 × MIC	–	0.07	0.191	2.56	–	2.92	2.555	7.53
0.0094	0.5 × MIC	–	2.80	0.627	1.31	–	3.53	18.58	–2.68
0.0188	1 × MIC	–	3.40	8.333	–2.01	–	4.44	8.429	–2.44
0.0752	4 × MIC	–	7.05	0.515	–1.70	–	8.62	0.607	–1.68
0.1504	8 × MIC	–	8.19	0.322	–3.40	–	15.82	0.002	–1.68
0.3008	16 × MIC	–	15.32	0.037	–3.12	–	19.44	0.101	–1.68
0.6016	32 × MIC	–	20.48	0.033	–2.66	–	39.15	0.028	–1.68

k_g, bacterial growth rate in the absence of antibiotic; k_{elb}, bacterial elimination rate; z, constant used to fit the initial lag phase for the growth; a, constant used to fit the phenotypic tolerance phenomenon.

antimicrobial resistance problem is self-medication with freely available antibiotics. Also, their uses as growth promoters in food animals such as cattle, pigs and chickens have produced the emergence of multi-resistant strains (Sparo and Schell, 2011). The search for new strategies for the treatment of bacterial infections has led to consider the role of strengthened interaction among microbiology, pharmacology and immunology.

Many methodologies to quantify and evaluate the in vitro antibacterial effects of bacterial killing curves have been described (Andraud et al., 2011; Corvaisier et al., 1998; Firsov et al., 1997; Guerillot et al., 1993; Maya et al., 2000; Mueller et al., 2004) but the treatment and interpretation of the obtained results remain a problem to be solved. Many results of such trials are lost because their interpretation is performed by a single method or using a single efficiency parameter type.

In our case, it is important to know how the CS, which has been shown to have time-dependent antibacterial activity (Siroski et al., 2009), can enhance the effectiveness of antibiotic with concentration-dependent activity as ciprofloxacin. In this assay the antibacterial activity of different concentrations of ciprofloxacin on *E. coli* (ATCC 25922) can be expressed in two ways: bacterial elimination rate and magnitude of elimination of viable bacteria at the end of the trial. According to these two aspects, two areas are distinguished in the graphs of the curves of death performed in this assay as presented in Fig. 6.

First, the theoretical phase of bacterial elimination defined between the start time (t_0) and the time at which the slope of bacterial clearance finish (t_a), in which the reduction of viable bacteria is observed. In this phase was detected that the bacterial elimination rate was increased in the presence of CS.

A second phase is defined between t_n and the end time (t_z) showed that the bacterial elimination rate decreased with time, and that a substantial fraction of the bacteria remained viable at the end of the assay, denominating this one phenotypic tolerance phase. During this phase, the presence of CS increased the efficacy of ciprofloxacin, which was expressed as a lower number of viable bacteria present at the end of the assay. Phenotypic tolerance phenomenon of ciprofloxacin can be explained because this antibiotic has been characterized by its extraordinary killing effect on bacteria which are in a logarithmic phase of growth (Zeiler and Grohe, 1984), and during antibiotic exposure, an enrichment of the fraction of slowly growing bacteria occurs, and thus a decrease in the overall bacteria mortality (Tuomanen et al., 1986; Wiuff et al., 2005).

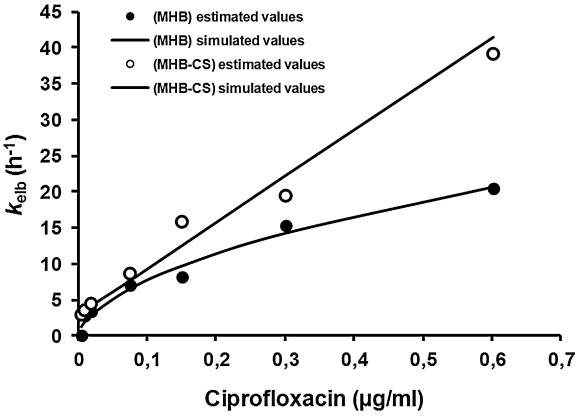

Fig. 5. Relationship between ciprofloxacin concentrations and the estimated bacterial elimination rate (k_{elb}) in Müller–Hinton broth (MHB) (●) and Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS) (○). Experimental data observed in MHB were fitted using Equation 3, and the observed in MHB-CS were fitted using Equation 4.

Table 5
Pharmacodynamic parameters estimated by fitting of the dose–response relationship between ciprofloxacin concentrations and the estimated bacterial elimination rate (k_{elb}) in Müller–Hinton broth (MHB) and Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS). Experimental data observed in MHB were fitted using Equation 3, and the observed in MHB-CS were fitted using Equation 4.

MHB		MHB-CS	
k_{max} (h^{-1})	165.5	a	64.0
C_{50} (μg/ml)	15.7	b	2.91
n	0.60		

k_{max}, is the maximum bacterial elimination rate; C_{50}, concentration of ciprofloxacin necessary to produce 50% of the maximum effect; n, Hill coefficient; a, is the slope of the line; b, is the y-intercept of the line.

Table 6
Antibacterial activity of ciprofloxacin against *Escherichia coli* (ATCC 25922) expressed as time required for the bacterial viable count to reduce by 50% (t_r) at several concentrations in Müller–Hinton broth (MHB) and Müller–Hinton broth/serum of broad-snouted caiman 50:50 (MHB-CS). The fasted reduction of viable count in the presence of serum of broad-snouted caiman are in bold.

Ciprofloxacin		t_r (min)		
μg/ml		MHB	MHB-CS	*diff.*
0.0047	0.25 × MIC	573.4	**14.2**	559.2
0.0094	0.5 × MIC	14.9	**11.8**	3.1
0.0188	1 × MIC	12.2	**9.4**	2.9
0.0752	4 × MIC	5.9	4.8	1.1
0.1504	8 × MIC	5.1	2.6	2.4
0.3008	16 × MIC	2.7	2.1	0.6
0.6016	32 × MIC	2.0	1.1	1.0

t_r is the time required for the bacterial viable count to reduce by 50%; *diff* is the difference between MHB values and MHB-CS values.

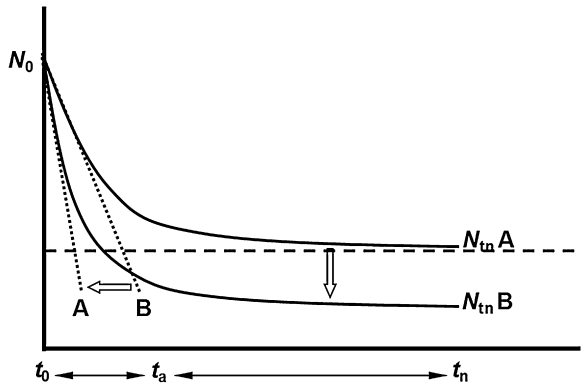

Fig. 6. Antibacterial activity of ciprofloxacin expressed as the rate and the magnitude of bacteria elimination in standard culture medium and in the presence of caiman serum. N_0 is the initial inoculum, N_{tn} A and N_{tn} B are the number of bacteria resulting from the exposure to antibiotic at the end of the experiment in a standard culture medium and in the presence of caiman serum respectively, A and B are the slopes of the bacteria elimination in the presence of caiman serum and in a standard culture medium, t_0 and t_n are the start time and the end time respectively. The vertical and horizontal arrows indicate that from a ciprofloxacin concentration in the presence of caiman serum, the viable bacteria decrease and the bacteria elimination rate increase respecting the observed in a standard culture medium, the area between t_0 to t_a is the theoretical bacteria elimination phase and the area between t_a to t_n is the theoretical phenotypic tolerance phase.

The phenotypic tolerance determines the effectiveness of ciprofloxacin based on the number of viable bacteria at the end of the assay could be erroneously evaluated, because similar efficacy could be estimated for different concentrations of the antibiotic.

So we believe that the in vitro activity of an antibiotic cannot be adequately assessed and quantified in a single way, but different methods should be used in order to obtain more information of the antibacterial activity.

The evaluation of the efficacy expressed in terms of reduction of number of viable bacteria showed that in the presence of CS, all ciprofloxacin concentrations except 0.0047 μg/ml, achieved in reducing the number of viable bacteria in 99.9% at the end of assay regarding size of the initial inoculum (Fig. 2B, Table 2), while in the absence of CS this efficacy was observed only at concentrations of 0.0188 μg/ml and 0.0752 μg/ml (Fig. 2A, Table 1).

When the efficacy was evaluated in terms of reduction of the bacterial biomass, it was found that the synergistic bactericidal activity between CS and ciprofloxacin on *E. coli* (ATCC 25922) was manifested at low concentrations of the antibiotic: 0.0047–0.0188 μg/ml (Fig. 3 and Table 3). The synergism between CS and ciprofloxacin was also evident with high values of bacterial elimination constant (k_{elb}) in the presence of CS (Table 4), resulting in lower values of bacterial reduction times (t_r), this being more marked between concentrations of 0.0047–0.0188 μg/ml (Table 6). These two findings allow to infer that the role of the immune factors present in CS not only to increase the effectiveness of the antibiotic but also to complement its activity at sub-inhibitory concentrations ($0.25 \times$ MIC and $0.5 \times$ MIC) as presented in Fig. 2.

The sub-inhibitory concentrations determine the reduction of the rate of bacterial growth and loss of viability. Under these conditions, bacteria present morphological alterations indicating disturbances in their metabolism and sensitization to host defense mechanisms (Lorian and Waluschka, 1972).

An important finding is the linear relationship between k_{elb} values and concentrations of ciprofloxacin in the presence of CS, wherein the bacterial kill-rate was increased in proportion to increasing concentrations of ciprofloxacin, while this kill-rate showed a tendency to diminish in the absence of CS (Fig. 5). The in vitro antibacterial activity of broad-snouted caiman plasma against *E. coli* exhibits time dependent inhibition growth (Siroski et al., 2009), thus the joint activity of sub-inhibitory concentrations of ciprofloxacin and humoral immune factors present in CS determine an increase in bacterial elimination observed in this assay.

We suggest that the alternative activity (Siroski et al., 2010) and classical pathway of complement by natural antibodies bound to both Gram-negative and Gram-positive bacteria can inhibit bacterial growth by lysis (Zhou et al., 2007). According to crocodilians lifestyle, they are exposed to apparent recurrent bacterial infection, so the serum levels of acute phase proteins and complement (C3) could be increased. As a consequence high levels of complement-derived proteolytic products could explain synergic effect of CS and ciprofloxacin on *E. coli* (ATCC 25922).

The detection of new components obtained from natural products encourages the use of wild ecosystems sustainably and they could become to see themselves as new resources for development of biomolecular compounds with therapeutic properties for human and veterinary medicine. This initial characterization allows inferences about the future evaluation of the applicability of serum derived from this species, as nonspecific complement antimicrobial therapy in animals of zootechnical interest. The transfer of these results would be applicable experimentally in domestic and wild species that are under different management programs. These findings generate an enhancement of not only the resource but also the ecosystem where they belong. Currently *Caiman latirostris* are being subject to sustainable management programs with an important social impact. Thus, the detection of derived components would be collaborated to the essential requirements applied to the sustainable use of natural resources based on long-term sustainability of the ecosystem and the social environment that surrounds it.

Acknowledgements

This work was supported by the funds of Universidad Nacional del Litoral, Argentina; Curso de Acción para la Investigación y Desarrollo (CAI + D) (PI-33-164).

References

Andraud, M., Chauvin, C., Sanders, P., Laurentie, M., 2011. Pharmacodynamic modeling of in vitro activity of marbofloxacin against Escherichia coli strains. Antimicrobial Agents and Chemotherapy 55, 756–761.

Baggot, J.D. (Ed.), 2001. The Physiological Basis of Veterinary Clinical Pharmacology. Blackwell Science, Oxford.

Blondeau, J.M., 2009. New concepts in antimicrobial susceptibility testing: the mutant prevention concentration and mutant selection window approach. Veterinary Dermatology 20, 383–396.

Britton, A., Diamond, G., Laube, D., Kaisser, V., 2002. Antimicrobial activity in the blood of the saltwater crocodile (*Crocodylus porosus*). In: Crocodiles. Proceedings of the 16th Working Meeting of the Crocodile Specialist Group. IUCN, Gland, Switzerland, p. 177.

CLSI, Clinical and Laboratory Standards Institute, 2008. Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents; Approved Guideline, vol. 28, third ed. Document M37-A3. Wayne, Pennsylvania, USA.

Corvaisier, S., Maire, P., Bouvier d'yvoire, M., Barbaut, X., Bleyzac, N., Jelliffe, R., 1998. Comparisons between antimicrobial pharmacodynamic indices and bacterial killing as described by using the Zhi model. Antimicrobial Agents and Chemotherapy 42, 1731–1737.

De Leo, F.R., Diep, B.A., Otto, M., 2009. Host defense and pathogenesis in *Staphylococcus aureus* infection. Infectious Disease Clinics of North America 23, 17–34.

Firsov, A., Sergey, N., Vostrov, S., Shevchenko, A., Cornaglia, G., 1997. Parameters of bacterial killing and regrowth kinetics and antimicrobial effect examined in terms of area under the concentration-time curve relationships: action of ciprofloxacin against *E. coli* in an in vitro dynamic model. Antimicrobial Agents and Chemotherapy 41 (6), 1281–1287.

Foster, T.J., 2004. The *Staphylococcus aureus* "superbug". Journal of Clinical Investigation 114, 1693–1696.

Guerillot, F., Carret, G., Flandrois, J., 1993. Mathematical model for comparison of time-killing curves. Antimicrobial Agents and Chemotherapy 37 (8), 1685–1689.

Hoffmann, J., Kafatos, F., Janeway, C.A., Jr., Ezekowitz, R.A.B., 1999. Phylogenetic perspectives in innate immunity. Science 284, 1313–1318.

Katie, L., Davies, R., Threlfall, E., 2005. Mechanism of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. International Journal of Antimicrobial Agents 25, 358–373.

Kays, M., Denys, G., 2001. Fluoroquinilone susceptibility, resistance and pharmacodynamics versus clinical isolates of *Streptococcus pneumoniae* from Indiana. Diagnostic Microbiology and Infectious Disease 40, 193–198.

Kristian, S.A., Timmer, A., Liu, G.Y., Lauth, X., Sal-Man, N., Rosenfeld, Y., et al., 2007. Impairment of innate killing mechanism by bacteriostatic antibiotics. The FASEB Journal 21, 1107–1116.

Levy, O., 2000. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. Blood 96, 2664–2672.

Liu, P., Muller, M., Derendorf, H., 2002. Rational dosing of antibiotics: the use of plasma concentrations versus time concentrations. International Journal of Antimicrobial Agents 19, 285–290.

Lorian, V., Waluschka, A., 1972. Blood cultures showing aberrant forms of bacteria. American Journal of Clinical Pathology 57 (3), 406–409.

Mattie, H., 1994. Pharmacokinetic and pharmacodynamic models of antimicrobial drugs *in vivo and in vitro*. Infection 22, 377–378.

Maya, J., Chan, C., Kinga, A., Williams, L., French, G., 2000. Time-kill studies of tea tree oils on clinical isolates. Journal of Antimicrobial Chemotherapy 45, 639–643.

Mehrzad, J., Duchateul, L., Burvenich, C., 2009. Phagocytic and bactericidal activity of blood of milk-resident neutrophyls against *Staphylococcus aureus* in primiparus and multiparus cows during early lactation. Veterinary Microbiology 134, 106–112.

Merchant, M.E., Roche, C., Elsey, R.M., Prudhomme, J., 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). Comparative Biochemistry and Physiology B 136, 505–513.

Mueller, M., De la Peña, A., Derendorf, H., 2004. Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC. Antimicrobial Agents and Chemotherapy 48, 369–377.

NCCLS, National Committee for Clinical Laboratory Standards, 1992. Methods for Determining Bactericidal Activity of Antimicrobial Agents: Tentative Guideline M26-T. Villanova, PA.

Odenholt, I., 2001. Pharmacodynamic effects of subinhibitory antibiotic concentrations. International Journal of Antimicrobial Agents 17, 1–8.

Piras, C., Soggiu, A., Bonizzi, L., Gaviraghi, A., Deriu, F., De Martino, L., et al., 2012. Comparative proteomics to evaluate multi drug resistance in *Escherichia coli*. Molecular BioSystems 8 (4), 1060–1067.

Piras, C., Soggiu, A., Greco, V., Cassinotti, A., Maconi, G., Ardizzone, S., et al. 2014. Serum protein profiling of early and advanced stage Crohn's disease. EuPA Open Proteomics 3, 45–59.

Siroski, P.A., Piña, C., Larriera, A., Merchant, M., Di Conza, J., 2009. Plasma activity of the broad-snouted caiman (*Caiman latirostris*). Zoological Studies 48, 238–242.

Siroski, P.A., Merchant, M., Parachú Marcó, M.V., Piña, C., Ortega, H.H., 2010. Characterization of the serum complement activity of the broad-snouted caiman *Caiman latirostris* (Crocodilia: Alligatoridae). Zoological Studies 49, 64–70.

Sparo, M., Sanchez Bruni, S.F. 2012. Approach to optimise the treatment of bacterial infections: combined antimicrobial therapy with enhancers of innate immunity. Clinical and Experimental Pharmacology 2, e106.

Sparo, M., Schell, C., 2011. Bacterias y alimentos. In: "Temas de Zoonosis 5", Asociación Argentina de Zoonosis, Vol. 43, pp. 379–386.

Tourn, S., Imhof, A., Costa, A., Von Finck, C., Larriera, A., 1994. Colecta de sangre y procesamiento de muestras en *Caiman latirostris*. In: Memorias del IV Workshop Sobre Conservación y Manejo del Yacaré Overo (*Caiman latirostris*). "La Región" – Fundación Banco Bica – Santo Tomé, Santa Fe, Argentina. pp. 25–30.

Tuomanen, E., Cozens, R., Tosch, W., Zak, O., Tomasz, A., 1986. The rate of killing of *E. coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. Microbiology (Reading, England) 132, 1297–1304.

White, R.L., 2001. What *in vitro* models of infection can and cannot do?: potential drawbacks of *in vitro* pharmacodynamic studies. Pharmacotherapy 21, 291–301.

Wiuff, C., Zappala, R.M., Regoes, R.R., Garner, K.N., Baquero, F., Levin, B.R., 2005. Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. Antimicrobial Agents and Chemotherapy 49, 1483–1494.

Wright, G.D., 2010. Q&A: antibiotic resistance: where does it come from and what can we do about it? BMC Biology 8, 123.

Zeiler, H.J., Grohe, K., 1984. The *in vitro* and *in vivo* activity of ciprofloxacin. European Journal of Clinical Microbiology 3, 339–343.

Zhou, Z.-H., Zhang, Y., Hu, Y.-F., Wahl, L.M., Cisar, J.O., Notkins, A.L., 2007. The broad antibacterial activity of the natural antibody repertoire is due to polyreactive antibodies. Cell Host Microbe 1, 51–61.

Zippel, K.C., Lillywhite, H.B., Mladnich, C.R., 2003. Anatomy of the crocodilian spinal vein. Journal of Morphology 258, 327–335.