Universitat Autònoma de Barcelona

New insights on the treatment of respiratory diseases caused by *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* in pigs with marbofloxacin

Tesi doctoral presentada per Carles Vilalta Sans per accedir al grau de Doctor en Veterinària dins del programa de Doctorat en Farmacologia del Departament de Farmacologia, de Terapèutica i de Toxicologia de la Facultat de Veterinària de la Universitat Autònoma de Barcelona (UAB), sota la direcció del Dr. Lorenzo José Fraile Sauce i la tutoria del Dr. Carles Cristòfol Adell.

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FACULTAT DE VETERINÀRIA DE LA UAB

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I per tal que consti als afectes oportuns, signen el present certificat a Bellaterra, a deu d’octubre de 2014.

Dr. Lorenzo José Fraile Sauce

Dr. Carles Cristòfol Adell

Agraïments

“A scientific man ought to have no wishes, no affections, a mere heart of stone”
Charles Darwin.

Els sentiments d’alegria i tristor es barregen. L’alegria de saber que després de redactar aquesta tesi recuperaré part del meu temps, però amb la tristor que produeix constatar com s’acaba una de les millors etapes de la meva vida. Deu ser el que anomenen el síndrome d’Estocolm.

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No sé si un home de ciència ha de tenir un cor de pedra. El que es ben segur es que si tothom tingués un cor de pedra no hi hauria homes de ciència.

Summary

Marbofloxacin (MB) is a third generation fluoroquinolone widely used in different species to treat mainly respiratory infections. This antimicrobial possesses a wide spectrum of activity that includes two of the main respiratory pathogens associated with the porcine respiratory disease complex (PRDC), *Actinobacillus pleuropneumoniae* (APP) and *Haemophilus parasuis* (HP). APP is the causative agent of porcine pleuropneumonia and this bacterium can remain in the tonsils of asymptomatic pigs for a long period maintaining the disease in the herd. HP is a colonizer of the upper respiratory tract of pigs and the causative agent of Glässer's disease in swine. One of the ways to cope with these diseases is to treat the affected animals with fluoroquinolones.

A great deal of information is now available on the literature about the pharmacokinetics (PK) and pharmacodynamics (PD) relationships for fluoroquinolones. The two main PK-PD parameters used for fluoroquinolones are the relationship between the PK parameters, the observed maximum drug concentration (C_{max}) in serum and the area under the curve (AUC) with the PD parameter the minimum inhibitory concentration (MIC).

The present thesis aimed to expand the knowledge on the use of MB to treat and control two of the most common agents of the PRDC which are sensitive to fluoroquinolones, APP and HP, and the way that PK-PD relationships can help with dose optimization.

In the first study of this thesis (study I), the penetration into tonsils of MB was assessed in young fattening pigs. Two different dosages were used to treat the animals: 2 mg/kg bw every 24 hours during 3 days (P1 group) and 4 mg/kg bw every 48 hours two times (P2 group). MB achieved a mean concentration in tonsils of 0.5 and 0.7 µg/g 24 hours after the last administration in groups P1 and P2, respectively. MB achieved a tonsillar concentration three times greater

than the plasma concentration whatever the dose administered 24 hours after the last administration. A ratio between the mean tonsillar concentration of MB for both doses (0.5 and 0.7 µg/g) and its MIC_{90} for APP (0.03 µg/mL) was calculated. These ratio values were 16.6 and 23.3 for P1 and P2 group, respectively and were also above the threshold established for PK-PD efficacy parameters in the case of fluoroquinolones. Later research after the publication of this study and using the same methodology but with the dose of 8 mg/kg in one shot showed a mean MB concentration of 0.73 µg/mL and 2.27 µg/g in plasma and tonsil respectively, giving as a result a ratio MB_{tissue}/MB_{plasma} of 3 at 24 hours after the MB administration in accordance with the previous results. Using the same MIC_{90} value as described the ratio MB_{tonsil}/MIC_{90} for this latter dose was 75 suggesting that this last higher dose could also be suitable to eradicate APP from the tonsil. Microbiological studies carried out on the farm showed that the prevalent infectious APP strain of the farm had a MB MIC value of 0.25 µg/mL. Besides, in this non-published microbiological study APP was still found viable in four out of ten tonsils of the 8 mg/kg MB group analysed indicating that APP was not eradicated from the tonsil even though that according to the PK-PD information related to the MB_{tonsil}/MIC ratio it should have been enough MB to kill the bacteria.

Study II focused on the colonization of HP after the treatment with MB and how this treatment may also affect the HP strain variability. First, it was selected the posology regimen that was able to decrease the detection of this bacteria at nasal mucosa between three posology regimens used frequently by clinicians (three doses of 2 mg/kg bw every 24 hours, two doses of 4 mg/kg bw every 48 hours and 8 mg/kg bw in one single shot). The three MB treatments reduced significantly ($p<0.05$) the nasal colonization by HP as compared to control animals. Moreover, HP was not detected at all in the nasal cavities of piglets after administering the highest dose. Secondly, it was studied the effect of a dose of 8 mg MB/kg bw in one shot on the strains population of HP in a farm with clinical cases of Glässer's disease using a longitudinal study. It was observed a statistically significant reduction of colonization by HP during the first week after treatment. On the other hand, a clear relationship between the

MIC of the different strains, their putative virulence and the treatment group from which they were isolated was not detected. Finally, in this particular case, the effect induced by the antibiotic treatment on the bacteria population seems to be transitory because it was observed a diverse HP population (with high and low MIC) seven days after finishing the treatment. This result clearly suggests that the population dynamics of bacteria is affected by many factors not only the selection pressure coming from the antibiotic treatment.

The last study (Study III) evaluated the theoretical clinical outcome of three MB posology regimens in two groups of pigs (weaners and fatteners) for the treatment of APP and HP infection and the appearance of resistant bacteria due to the antibiotic treatment. The probability of target attainment (PTA) for pharmacokinetic-pharmacodynamics (PK-PD) ratios associated with clinical efficacy and with the appearance of antimicrobial resistance for fluoroquinolones at each MIC or mutant prevention concentration (MPC) were calculated, respectively. The cumulative fraction of response (CFR) was calculated for the three posology regimens against APP and it ranged from 91.12 % to 96.37 % in weaners and from 93 % to 97.43 % in fatteners. In the case of HP, the CFR ranged from 80.52 % to 85.14 % in weaners and from 82.01 % to 88.49 % in fatteners. Regarding to the PTA of the PK-PD threshold associated with the appearance of antimicrobial resistance, results showed that MB would prevent resistances in most of the animals up to the MPC value of 1 µg/mL.

Resum

La marbofloxacina (MB) és una fluoroquinolona de tercera generació àmpliament usada en diferents espècies per tractar sobretot infeccions respiratòries. Aquest antibiòtic posseeix un ampli espectre d'activitat que inclou dos dels principals patògens associats al complexe respiratori porcí (CRP), *Actinobacillus pleuropneumoniae* (APP) i *Haemophilus parasuis* (HP). APP és l'agent etiològic de la pleuropneumònia porcina i pot romandre a les tonsil·les dels porcs sense mostrar cap mena de símptoma durant llargs períodes de temps prolongant així la malaltia dins de l'explotació. HP és una bactèria colonitzadora del tracte respiratori superior dels porcs i l'agent etiològic de la malaltia de Glässer en la mateixa espècie. Una de les maneres per combatre aquestes malalties es tractar els animals infectats amb fluoroquinolones.

Actualment, hi ha una gran quantitat d'informació existent en la literatura sobre les relacions entre la farmacocinètica (PK) i la farmacodinàmica (PD) de les fluoroquinolones. Els dos principals paràmetres PK-PD descrits per les fluoroquinolones són les relaciones existents entre els paràmetres PK, la concentració màxima observada en sèrum de l'antibiòtic (C_{max}) i l'àrea sota la corba (AUC), amb el paràmetre PD concentració mínima inhibitòria (CMI).

L'objectiu de la present tesi doctoral era expandir el coneixement sobre l'ús de la MB per tractar i controlar dos dels agents més comuns del CRP que són sensibles a les fluoroquinolones, APP i HP, i la manera com les relacions PK-PD poden ajudar en l'optimització de la dosi.

En el primer estudi d'aquesta tesi (estudi I), es va avaluar la penetració de la MB en les tonsil·les de porcs en la fase inicial d'engreix. Es van fer servir dues dosificacions diferents per tractar els animals: 2 mg/kg pv cada 24 h durant 3 dies (grup P1) i 4 mg/kg pv dues vegades, una cada 48 h (grup P2). La concentració mitja de la MB 24 hores després de la última administració va ser de 0.5 µg/g en el grup P1 i 0.7 µg/g en el grup P2. La MB va assolir una

concentració a la tonsil·la tres vegades superior que la del plasma a les 24 h després de la última administració sense importar quina fou la dosi administrada. Posteriorment es va calcular la ràtio entre la concentració mitja de la MB en ambdues dosis (0.5 i 0.7 μg/g) i el CMI_{90} de APP (0.03 μg/mL). El resultat d'aquestes ràtios va ser de 16.6 pel grup P1 i 23.3 pel grup P2 i en ambdós casos el resultat va estar per sobre el llindar d'eficàcia establert pels paràmetres PK-PD per les fluoroquinolones. En una recerca posterior a la publicació d'aquest estudi i fent servir la mateixa metodologia però amb la dosi de 8 mg/kg en una sola aplicació es va poder observar una concentració mitja de la MB de 0.73 μg/mL en plasma i 2.27 μg/g en la tonsil·la, donant una ràtio MB_{teixit}/MB_{plasma} a les 24 hores després de l'administració de la MB igual a tres en concordança amb els resultats comentats anteriorment. Utilitzant el mateix valor de la CMI_{90}, la ràtio $MB_{tonsil·la}/CMI_{90}$ per aquesta última dosi fou de 75 suggerint que aquesta dosi més alta podria resultar idònia per eradicar APP de la tonsil·la Estudis microbiològics portats a terme en la granja van mostrar que la soca infectiva predominant d'APP en l'explotació tenia un valor de CMI de 0.25 μg/mL enfront a la MB. A més a més, en aquest estudi microbiològic no publicat es va poder trobar que APP encara era viable en quatre de les deu tonsil·les analitzades en la dosi de 8 mg/kg indicant que APP no va poder ser eradicat tot i que segons la informació PK-PD relacionada amb la ràtio $MB_{tonsil·la}/CMI$ hi hauria hagut suficient MB per matar la bactèria.

L'estudi II es va centrar en la colonització dels porcs per part de HP després del tractament amb MB i com aquest tractament podia afectar la variabilitat de les diferents soques de HP. Primer, es va seleccionar el tipus de posologia que fos capaç de reduir la detecció d'aquesta bactèria a la mucosa nasal entre els tres tipus de posologies més freqüents entre els clínics (tres dosis de 2 mg/kg pv cada 24 h, dues dosis de 4 mg/kg pv cada 48 h i 8 mg/kg pv en una sola administració). Els tres tractaments van reduir significativament ($p<0.05$) la colonització nasal per part de HP quan es van comparar amb el grup control. A més a més, HP no es va poder detectar en les cavitats nasals dels animals tractats amb la dosi més alta. Segon, es va estudiar l'efecte de la dosi de 8 mg de MB/kg pv en una única administració sobre la població de soques de HP en

una granja amb casos clínics de la malaltia de Glässer mitjançant un estudi longitudinal. Com a resultat es va observar una reducció estadísticament significativa de la colonització de HP fins a una setmana després del tractament. Per altra banda, no es va poder detectar una clara relació entre la CMI de les diferents soques, la seva possible virulència i el grup en el que van ser aïllades. Finalment, i en aquest cas en particular, l'efecte induït pel tractament antibiòtic sobre la població bacteriana va semblar ser transitori ja que set dies després d'acabar el tractament es va poder observar una diversitat en la població de HP (amb CMI altes i baixes). Aquest resultat clarament suggereix que la dinàmica de la població de HP està afectada no només per la selecció provinent del tractament antibiòtic sinó per altres factors.

En l'últim estudi (estudi III) es van avaluar el resultat clínic teòric en dos grups de porcs (en garrins i porcs d'engreix) de les tres dosificacions de MB usades pel tractament de les infeccions de APP i HP i l'aparició de bactèries resistents degut al tractament antibiòtic. Aquesta avaluació va a ser portada a terme mitjançant el càlcul de la probabilitat d'assoliment de l'objectiu (PAO) de les ràtios de la relació farmacocinètica-farmacodinàmia (PD-PD) associades amb l'eficàcia clínica del tractament i amb l'aparició de resistències antibiòtiques de les fluoroquinolones per cada CMI o concentració de prevenció de mutants (CPM). Es va calcular la fracció acumulativa de resposta (FAR) de les tres posologies contra APP i els resultats van anar des de 91.12 % fins a 96.37% en garrins i des de 93 % fins 97.43 % en els porcs d'engreix. En el cas de HP, la FAR va estar entre 80.52 % i 85.14 % en garrins i des de 82.01 % fins a 88.49 % en porcs d'engreix. En referència a la PAO del llindar associat als paràmetres PK-PD d'aparició de resistències antimicrobianes els resultats van mostrar que la MB podria prevenir l'aparició de resistències en la majoria dels animals fins a nivells de CPM de 1 µg/mL.

Resumen

La marbofloxacina (MB) es una fluoroquinolona de tercera generación ampliamente usada en diferentes especies principalmente en el tratamiento de infecciones respiratorias. Este antibiótico posee un amplio espectro de actividad que incluye dos de los principales patógenos asociados al complejo respiratorio porcino (CRP), *Actinobacillus pleuropneumoniae* (APP) y *Haemophilus parasuis* (HP). APP es el agente etiológico de la pleuropneumonia porcina y puede permanecer en las tonsilas de los cerdos durante largos períodos de tiempo sin mostrar ningún síntoma prolongando así la enfermedad dentro de la explotación. HP es una bacteria colonizadora del tracto respiratorio superior de los cerdos y el agente etiológico de la enfermedad de Glässer en la misma especie. Una de las maneras de luchar contra estas enfermedades es tratar los animales infectados con fluoroquinolonas.

Actualmente, hay una gran cantidad de información existente en la literatura sobre las relaciones entre la farmacocinética (PK) y la farmacodinamia (PD) de las fluoroquinolonas. Los dos principales parámetros PK-PD descritos para las fluoroquinolonas son las relaciones existentes entre los parámetros PK, la concentración máxima del antibiótico observada en suero (C_{max}) y el área bajo la curva (AUC), con el parámetro PD concentración mínima inhibitoria (CMI).

El objetivo de la presente tesis doctoral era expandir el conocimiento sobre el uso de la MB para tratar y controlar dos de los agentes más comunes del CRP que son sensibles a las fluoroquinolones, APP y HP, y la manera como las relaciones PK-PD pueden ayudar en la optimización de la dosis..

En el primer estudio de esta tesis (estudio I), se evaluó la penetración de la MB en las tonsilas de cerdos en la fase inicial de engorde. Se utilizaron dos dosificaciones diferentes para tratar los animales: 2 mg/kg pv cada 24 h durante 3 días (grupo P1) y 4 mg/kg pv dos veces una cada 48 h (grupo P2). La concentración media de la MB 24 horas después de la última administración

fue de 0.5 µg/g en el grupo P1 y 0.7 µg/g en el grupo P2. La MB va alcanzó una concentración en la tonsila tres veces superior que la del plasma 24 h después de la última administración sin importar cuál fue la dosis administrada. Posteriormente se calculó la ratio entre la concentración media de la MB en las dos dosis (0.5 y 0.7 µg/g) y el CMI_{90} de APP (0.03 µg/mL). El resultado de estas ratios fue de 16.6 en el grupo P1 y 23.3 en el grupo P2 y en los dos casos el resultado estuvo por encima del umbral de eficacia establecido para los parámetros PK-PD para las fluoroquinolonas. En una investigación posterior a la publicación de este estudio y utilizando la misma metodología pero con la dosis de 8 mg/kg en una sola aplicación se observó una concentración media de MB de 0.73 µg/mL en plasma y 2.27 µg/g en la tonsila, dando como resultado de la ratio MB_{tejido}/MB_{plasma} a les 24 horas después de la administración de la MB igual a tres que concuerda con los resultados previamente comentados. Utilizando el mismo valor de la CMI_{90}, la ratio $MB_{tonsila}/CMI_{90}$ para esta última dosis fue de 75 sugiriendo que esta dosis más alta podría resultar idónea para erradicar APP de la tonsila Estudios microbiológicos llevados a cabo en la granja mostraron que la cepa infectiva predominante de APP en la explotación tenía un valor de CMI de 0.25 µg/mL frente a la MB. Además, en este estudio microbiológico no publicado se encontró que APP aún era viable en cuatro de las diez tonsilas analizadas para la dosis de 8 mg/kg indicando que APP no pudo ser erradicado aunque según la información PK-PD relacionada con la ratio $MB_{tonsila}/CMI$ habría habido suficiente MB para eliminar la bacteria.

El estudio II se centró en la colonización de los cerdos por parte de HP después del tratamiento con MB y como este tratamiento podía afectar la variabilidad de las diferentes cepas de HP. Primero, se seleccionó el tipo de posología que fuese capaz de reducir la detección de esta bacteria en la mucosa nasal entre los tres tipos de posologías más frecuentes entre los clínicos (tres dosis de 2 mg/kg pv cada 24 h, dos dosis de 4 mg/kg pv cada 48 h y 8 mg/kg pv en una sola administración). Los tres tratamientos redujeron significativamente ($p<0.05$) la colonización nasal por parte de HP cuando se compararon con el grupo control. Además, HP no se pudo detectar en las cavidades nasales de los animales tratados con la dosis más alta. Segundo, se

estudió el efecto de la dosis de 8 mg de MB/kg pv en una única administración sobre la población de cepas de HP en una granja con casos clínicos de la enfermedad de Glässer mediante un estudio longitudinal. Como resultado se observó una reducción estadísticamente significativa de la colonización de HP hasta una semana después del tratamiento. Por otro lado, no se pudo detectar una relación clara entre la CMI de las diferentes cepas, su posible virulencia y el grupo en el que fueron aisladas. Finalmente, y en este caso en particular, el efecto inducido por el tratamiento antibiótico sobre la población bacteriana pareció ser transitorio ya que siete días después de terminar el tratamiento se pudo observar una diversidad en la población de HP (con CMI altas y bajas). Este resultado claramente sugiere que la dinámica de la población de HP está afectada no solo por la selección proveniente del tratamiento antibiótico sino por otros factores.

En el último estudio (estudio III) se evaluaron el resultado clínico teórico en dos grupos de cerdos (en lechones y cerdos de engorde) de las tres dosificaciones de MB usadas para el tratamiento de les infecciones de APP y HP y la aparición de bacterias resistentes debido al tratamiento antibiótico. Esta evaluación fue llevada a cabo mediante el cálculo de la probabilidad de alcanzar el objetivo (PAO) de las ratios de la relación farmacocinética-farmacodinamia (PD-PD) asociadas con la eficacia clínica del tratamiento y con la aparición de resistencias antibióticas de las fluoroquinolones por cada CMI o concentración de prevención de mutantes (CPM). También se calculó la fracción acumulativa de respuesta (FAR) de las tres posologías contra APP y los resultados fueron desde 91.12 % hasta a 96.37% en lechones y desde 93 % hasta 97.43 % en los cerdos de engorde. En el caso de HP, la FAR estuvo entre 80.52 % y 85.14 % en lechones y desde 82.01 % hasta 88.49 % en cerdos de engorde. En referencia a la PAO del umbral asociado a los parámetros PK-PD de aparición de resistencias antimicrobianas los resultados sugirieron que la MB podría prevenir la aparición de resistencias en la mayoría de los animales hasta niveles de CPM de 1 µg/mL.

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List of abbreviations

ADME	Absorption, Distribution, Metabolism and Elimination
APP	*Actinobacillus pleuropneumoniae*
AUC	Area under the plasma concentration vs time curve
AUC/MPC	Relationship between the AUC and the MPC
AUC_{0-24}	Area under the plasma concentration vs time curve during the first 24 hours
AUC_{0-t} or AUC_{last}	Area under the plasma concentration vs time curve from 0 to the last measured point.
$AUC_{0-\infty}$ or AUC_{inf}	Area under the plasma concentration vs time curve from 0 to infinite
AUC_{ss} or AUC_{24}	Area under the plasma concentration vs time curve during 24 hours in a steady state
AUC/MIC	Relationship between the AUC and the MIC
CASFM	Comité de l'Antibiogramme de la Société Française de Microbiologie
CFR	Cumulative Fraction of Response
CLSI	Clinical and Laboratory Standards Institute
Cmax	Maximum plasmatic concentration
Cmax/MIC	Ratio between the maximum concentration in plasma and the MIC
Cmax/MPC	Ratio between the maximum concentration in plasma and the MPC
C_{ss}	Steady state concentration
EMA	European Medicines Agency
EUCAST	European Committee on Antimicrobial Susceptibility Testing
e.v.	Extravenous

F or F%	Bioavailability
FDA	Food and Drug Administration
FQ	Fluoroquinolone or Fluorquinolones
I	Indeterminate or intermediate, when talking about bacterial sensitivity to antimicrobials
IC	Inhibitory quotient
IR	Inhibitory ratio
i.v.	Intravenous
HP	*Haemophilus parasuis*
MB	Marbofloxacin
MBC	Minimum Bactericidal Concentration
MCS	Monte Carlo Simulations
MIC	Minimum Inhibitory Concentration
MIC50	Minimum Inhibitory Concentration for the 50% of the analyzed strains
MIC90	Minimum Inhibitory Concentration for the 90% of the analyzed strains
MPC	Mutant Prevention Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSW	Mutant Selection Window
P	Coefficient of partition
PAE	Post Antibiotic Effect
PALE	Post Antibiotic Leukocyte Enhancement
PD	Pharmacodynamics
PELF	Pulmonary Epithelial Lining Fluid
PK	Pharmacokinetics
PK-PD	Pharmacokinetic-Pharmacodynamic
PRDC	Porcine Respiratory Disease Complex

PRCv	Porcine Respiratory Coronavirus (PRCv).
PRRSv	Porcine Respiratory and Reproductive Syndrome virus
PRv	Pseudorabies virus
PTA	Probability of Target Attainment
R	Resistant, when talking about bacterial sensitivity to antimicrobials
S	Susceptible, when talking about bacterial sensitivity to antimicrobials
SIv	Swine Influenza virus
$\mathbf{T_{1/2}}$	Half-life of elimination
TAR	Target Attainment Rate
$\mathbf{T_{max}}$	Time in which the maximum plasma concentration is reached
$\mathbf{T_{MSW}}$	Time inside the mutant selection window
$\mathbf{T_{>MIC}}$	Drug concentration time above the MIC
$\mathbf{T_{>MPC}}$	Drug concentration time above the MPC
$\mathbf{T_{>MPC}/T_{MSW}}$	Ratio between the time above the MPC and the Time inside the mutant selection window
Vd	Volume of distribution
Vss	Volume of distribution in the steady state
vtaA	Virulence-associated trimeric autotransporter
$\mathbf{\lambda_z}$	Slope during the terminal phase

I. INTRODUCTION

Begin at the beginning and go on till you come to the end, then stop.

Lewis Carroll, Alice's Adventures in Wonderland

1. ANTIMICROBIAL THERAPY

1.1. Introduction

By the time that Paul Ehrlich, Ernest Duchesne and Alexander Fleming were doing their discoveries during the first half of the 20th century, they did not know that they were paving the way to the modern antimicrobial therapy. Ehrlich studied the interaction of different substances with infectious protozoans and bacteria, and one of his biggest achievements was to treat effectively syphilis in humans by using chemical compounds (arsphenamine and neoarsphenamine). Ernest Duchesne first and Alexander Fleming later, described the protective effect of mould (*Penicillium* species) in front of bacteria. However, Fleming was the one who successfully isolated the compound that killed the bacteria. That substance was the first antibiotic, penicillin. Since then, many other new antibiotics have been developed. However, concurrently to the development of antimicrobials, the appearance of resistant bacteria to antimicrobials highlighted the need for a responsible use of these drugs. Although, it was not until the last decade that scientist have done a significant effort to optimize antimicrobial therapy (Saga & Yamaguchi, 2009; Scaglione, 2002).

The concept of antimicrobial therapy involves three agents: the microorganism, the host (human or animal) and the drug. The interactions and the relationships between the three agents are summarized in the triangle below (Figure 1).

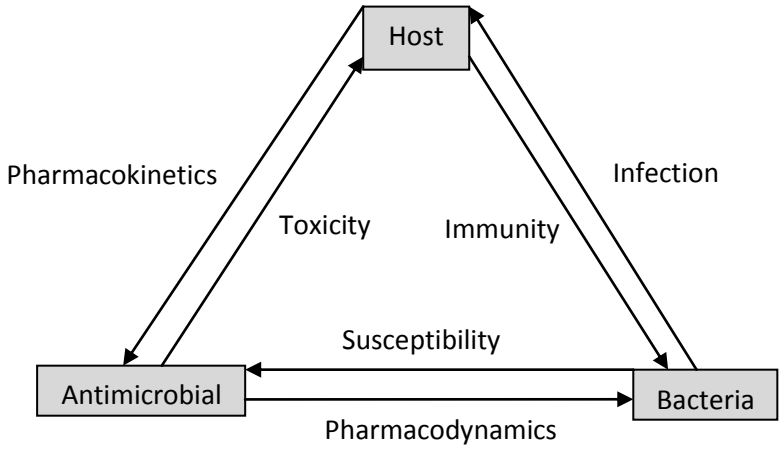


Figure 1. Graphical representation of the relationships between host, antimicrobial and bacteria.

All the relationships among these agents are important and largely studied by science although when it comes to calculate and design a therapeutic strategy against an infectious agent, the way that the body deals with the antimicrobial, drug pharmacokinetics (PK), the relationship between the drug concentration and its effect on the microorganisms, pharmacodynamics (PD), and the way how the PK and PD are related play an important role.

1.2. Pharmacokinetics

The pharmacokinetics describes, through mathematic concepts, the kinetics of a drug inside the organism and defines a pattern of the ADME process (Absorption, Distribution, Metabolism and Elimination) of each drug. In order to simplify the conceptualization of all the processes involved in the ADME process and analyze the experimental data, compartmental and non-compartmental models are used to describe and foresee the fate of the drug in the body. Compartmental modelization consists in describing the body as a sum of different compartments. Monocompartmental and bicompartmental models are the most frequently used to describe the pharmacokinetics of a drug. However, other multicompartmental models, such a three-compartment model, can fit better to describe the drug evolution in the body. Thus, tissues or compartments in which equilibrium is achieved soon after the drug administration – and from which this drug is redistributed to other sites – are referred to as “shallow” or “superficial” compartments; on the other hand, compartments in which equilibrium is achieved relatively late are referred to as “deep” compartments. Figure 2 summarizes in a general way the pharmacokinetic profile of the different compartments.

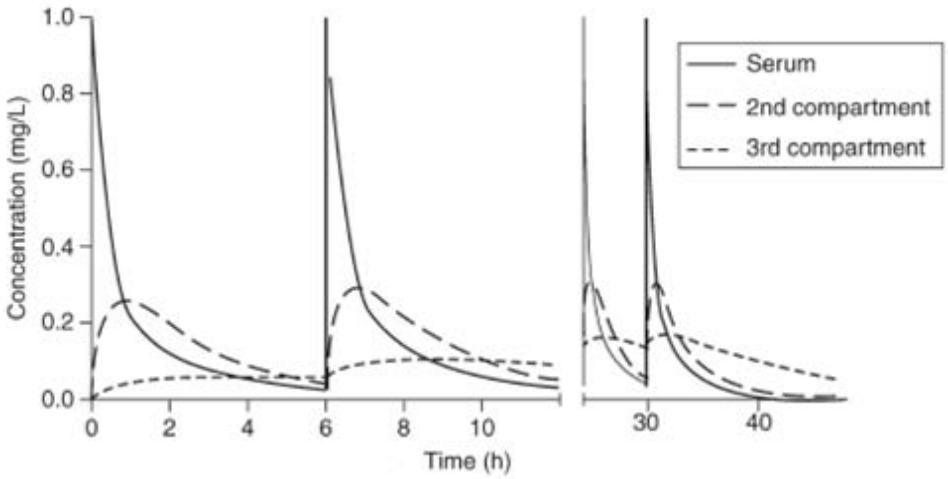


Figure 2. Schematic representation of the concentration-time relationship of a drug in a three compartment model in an every six-hour drug administration.

The most relevant pharmacokinetic parameters (C_{max}, t_{max}, AUC, bioavailability, volume of distribution, clearance and elimination half-life) have been described for many antimicrobial drugs in several species of veterinary interest. These parameters are necessary to establish the posology regimen for each particular species.

C_{max}: The C_{max} or peak concentration is the maximum concentration achieved during the dose interval (applied to extra vascular administration).

T_{max}: The time after the initial administration where the maximum concentration is reached.

C_{max} and T_{max} may be directly obtained from pharmacokinetic drug experiments on subjects.

AUC (area under the curve): The AUC is the area delimitated by the function concentration versus time and measures the drug exposure. The AUC usually is expressed taking into account the exposure time during the 24 hours after the administration ($AUC_{0-24)}$ or during a period of 24 hours in a steady state (AUC_{24}). However other expressions as AUC_{ss} (AUC at steady state), AUC_{0-t} (AUC to the last measured point; it can be found as $AUC_{last)}$ and $AUC_{0-\infty}$ (AUC from 0 to infinite, also called AUC_{inf}) can be found. The AUC is usually calculated by the trapezoidal rule; the total area is calculated by the sum of

individual trapezoids. However, if the number of sampling points is low the arithmetic rule will overestimate the AUC, for this reason a log-linear trapezoidal rule is used. Having a fair number of sampling points at the initial stage of the pharmacokinetic evaluation avoids underestimating the AUC.

Bioavailability: Represented as F or F%. It expresses the rate of drug which was absorbed and is ready to produce a systemic effect, comparing the exposure of the given extravenous (e.v.) administration route with the intravenous (i.v.) administration exposure which is assumed to be 100 % available (absolute bioavailability).

$$\frac{AC_{e.v.}}{AC_{i.v.}}$$

The bioavailability is specific for each pharmacological product and a given route of administration.

The relative bioavailability compares the exposure of two given formulations or two different routes of administration of the same formulation without taking into account the i.v. administration (Toutain & Bousquet-Mélou, 2004[d]).

Plasma Clearance: The plasma clearance calculates the drug removal of the body through elimination or metabolization of this drug, mainly done in the kidney and the liver. It is expressed as volume per time for each kilogram of body weight (L/h/kg or mL/min/kg) and represents the global ability of the body to remove a drug, relating this drug removal rate (amount per time) to the levels of the same drug in plasma.

$$Plasma\ clearance\ \frac{Total\ (body)rate\ of\ drug\ elimination}{Plasma\ concentration}$$

Plasma clearance is one of the most important parameters in pharmacokinetics and allows calculating the dosage rate along with the bioavailability and the steady-state therapeutic plasma concentration as expressed in the following equation:

$$Dosing\ rate \quad \frac{Plasma\ clearance \quad Therapeutic\ plasma\ concentration}{ioavailability}$$

In some circumstances and if the hepatic clearance is zero the plasma clearance is equal to the renal clearance and it can be mesurated/estimated measuring the total dug eliminated to the urine (Toutain & Bousquet-Mélou, 2004[a]).

Elimination half-life ($t_{1/2}$): Other called plasma half-life or half-life of the terminal phase and expresses the needed time to reduce the concentration in plasma by half during the terminal phase when the decrease of drug concentration only can be attributed to elimination.

The mathematical formula that describes elimination half-life is described as follows:

$$t_{1/2} \quad \frac{0.693}{\lambda_z}$$

where 0.693 is the natural logarithm of 2 and λ_z is the terminal phase slope.

Elimination half-life can also be related mathematically with clearance and volume of distribution using the following equation:

$$t_{1/2} \quad \frac{0.693 \quad Volume\ of\ distribution}{Plasma\ clearance}$$

Elimination half-life is very useful when it comes to calculate the right dose interval and predict drug accumulation in repeated administrations. In general terms, steady state is reached after 3-5 times the half-life, that means with short half-time drugs (12 hours or less) applied daily the steady state will be reach in two or three days. In short half-life time drugs the elimination half-life will be important to determine the dosage form to maintain the plasma concentration into the therapeutic interval. On the other hand, drugs with long half-time (more than 24 hours) will need more time to reach the steady state but also means

that accumulation issues will have to be taken into account to prevent plasma drug levels from crossing the toxicity threshold. Therefore, in this latter sort of drugs the administration of a loading dose to achieve the therapeutic threshold as soon as possible should be considered (Toutain & Bousquet-Mélou, 2004[b]).

Volume of distribution (V_d): This parameter corresponds to the ratio between the total amount of drug in the organism at a given time divided by the plasma concentration at that time.

$$V_d \quad \frac{\textit{Amount of drug in the organism at time } t}{\textit{Plasma concentration at time } t}$$

The main clinical use for the V_d is to calculate the loading dose (initial dose of a multiple dosage regimen) by calculating the V_{ss} (volume of distribution in the steady state) is the one that allows to estimate the loading dose:

$$\textit{Loading dose} \quad \frac{V_{ss} \;\; C_{ss}}{}$$

where C_{ss} is the foreseen plasma concentration at steady state and F is the bioavailability. The use of a loading dose can be useful when the C_{ss} wants to be reached fast (e.g. to treat an infection with an antibacterial drugs) and consequently the desired drug effect. Depending on the drug binding to proteins and tissues the value of V_d can be higher than the total amount of body water (Toutain & Bousquet-Mélou, 2004[c]).

Pharmacokinetic considerations and population pharmacokinetics

Most of the PK studies from which the data are derived in pharmacology are carried out in healthy adult animals. Nevertheless, some of the drug application and treatments are administered at early stages of life or in poor condition where some of the PK assumptions made previously can vary or can alter dose-concentration relationships, e.g. feed administration in anorexic animals or clearance modification in kidney affected animals. Some other factors such as

gestation, lactation or the presence of concomitant diseases or treatments can also affect the antimicrobial pharmacokinetics.

The population pharmacokinetics is a special type of PK analysis where the sources and correlates of variation are studied with larger numbers of individuals in a specific population than the conventional PK studies. Population PK tries to identify the factors that alter the dose-concentration relationship and the extent of these changes in order to modify the dosage if it was clinically necessary. Thus, this type of study give us information about how PK works in groups or subpopulations that otherwise are excluded, such as ill, nursery or elderly patients.

Compared to traditional PK analysis, population PK approach may include (FDA, 1999):

- Relevant PK information of the target population or subpopulation to be treated.
- The description and quantification of variability during drug development and evaluation.
- The explication that how these variability factors may affect and alter the PK of the drug.
- A quantitative estimation of the unexplained variability in the target population.

The use of population pharmacokinetics is more extended in human medicine for dose recommendation than in veterinary medicine. Some examples of population pharmacokinetics in humans are the published papers of Khachman *et al*. (2011) and Tanigawara *et al*. (2012). Khachman *et al.* (2011) studied the outcome, clinical and the likelihood of bacterial resistance, of different dosages regimes of ciprofloxacin in intensive care units in the treatment against the most common nosocomial pathogens. On the other hand, Tanigawara *et al*. (2012)

performed a prospective study to find the optimal dose of ganeroxacin to treat patients with respiratory infections.

Even though the scarcity of population PK studies in veterinary science, population PK has been gaining importance in the last years in veterinary studies (Guo *et al*., 2010; Black *et al*., 2014; Kinney *et al*., 2014; Zhao *et al*., 2014). Besides, population PK can be applied not only in the veterinary drug development process and therapeutics but also it can be applied to foresee tissue residues and estimate withdrawal intervals in food producing animals (Martin-Jimenez & Riviere, 1998). Briefly, the withdrawal time of a drug in veterinary medicine is the time necessary to reduce the drug levels in tissues and products (milk and eggs) below the maximum residue limits in 99% of the population of treated animals. Examples of the use of PK analysis in the prediction of withdrawal times can be found in research papers using flunixin and tetracyclines in cattle and pig (Wu *et al*., 2013; Lindquist *et al*., 2014).

Veterinarians usually treat large populations of animals. Thus, the use of population PK studies in veterinary medicine seems suitable to foresee and explore the behaviour of a drug at population level. However, these studies are not normally carried out and little information is available in the literature.

1.3. Pharmacodynamics

Pharmacodynamics studies the action of drugs on microorganisms or on specific receptors in the body to modify a physiological action. One of the difference between PD studies on mammalian cells/ tissues and on microbes is that the response in the former system is normally quantified as an enhancement or reduction of some component of cell or body function (smooth muscle contraction, decrease in body temperature, etc.), whereas pharmacodynamics on microorganisms establish threshold values (Minimum inhibitory concentration or MIC; minimum bactericidal concentration or MBC; or

mutant prevention concentration or MPC) to link the concentration of antibiotic with the growth of the microorganism population.

The *in vitro* PD parameter most widely used is the minimum inhibitory concentration (MIC). The MIC is determined under standard culture conditions either on a solid agar medium or, more usually, in liquid broth culture. It is defined as the lowest concentration of antimicrobial drug which prevents (as assessed by visual examination) visible microorganism growth. MIC is a simple, versatile, readily performed measure, which enables large numbers of microorganisms to be screened. Because MIC varies considerably between strains of a single organism, it is usual practice to measure MIC on many (up to several hundred) isolates and then compute the MIC_{50} and MIC_{90} values, the lowest concentration of antimicrobial that inhibits the growth of the 50% or the 90%, respectively, of the studied bacteria strains. To register an active ingredient for antimicrobial use, it is usual to use MIC_{90} rather than MIC_{100} values as the main pharmacodynamic parameter to establish its posology regimen. This is because, for any given population of microorganisms, there will commonly be a small percentage of isolates which are not susceptible, even to very high drug concentrations. MIC_{90} provides the best available indicator to link with PK data and establish a posology regimen to be applied under field conditions (Lees *et al*., 2004). It is important to take into account that the distribution of MICs in a wide range of isolates is not always normally distributed (Aliabadi *&* Lees, 2000).

The minimum bactericidal concentration (MBC) is the lowest concentration required to reduce the viability of an initial bacterial inoculums by ≥99.9 % after 24 hours of incubation at 37ºC. In other words, MBC is the lowest concentration with which the culture has been completely sterilized. The MBC is determined by subculturing each of the No Growth tubes in the MIC test to a solid antibiotic-free medium. Besides, the techniques to determine MBC have varied considerably over time and between laboratories, without being standarizated, therefore providing only a snapshot in time and place for a particular organism. Reproducibility of test results remains an ongoing problem in the inter and

intralaboratory standardization of such tests (Pankey & Sabath, 2004). For this reason the MIC, that has been standarizated, is preferred to compare bacteria susceptibility. Furthermore, MBC for bactericidal antimicrobials is very similar to MIC. A schematic representation on how MIC and MBC are obtained is presented in figure 3.

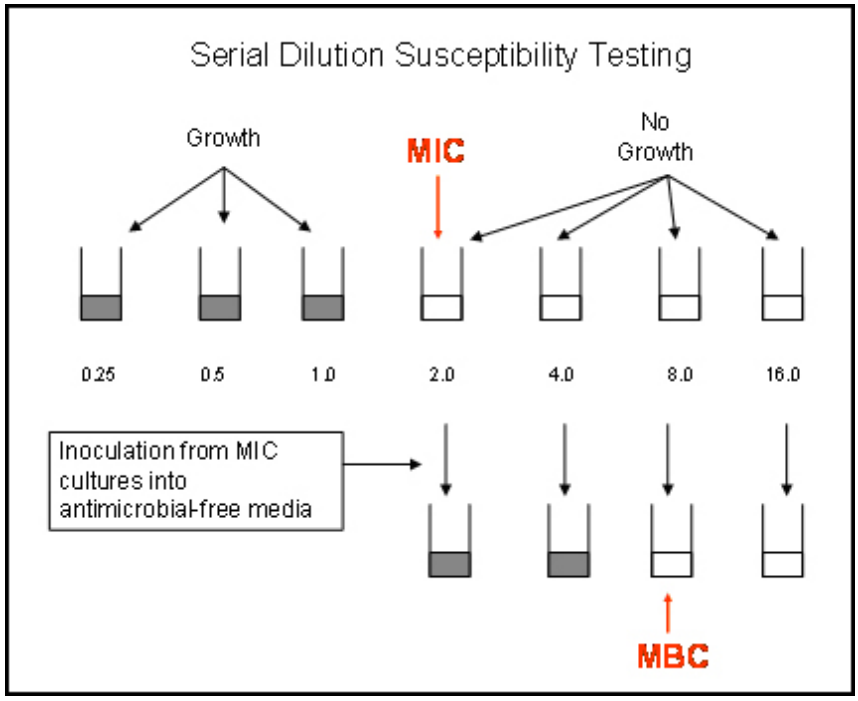


Figure 3. Schematic representation of MIC and MBC.

The mutant prevention concentration (MPC) is an in vitro PD value that relates the concentration with the appearance of resistances. Thus, it is defined as the MIC of the least susceptible single-step mutant. That means that growing above this concentration would require the presence of two or more mutations, which is rare (Zhao *et al*., 1997; Iseman, 1994). To ensure the presence of mutant subpopulations larger inocula of bacteria than the MIC determination (10^{10} cells in MPC against 10^4 -10^5 in MIC estimation) are used when is tested in agar or liquid medium (Dong *et al*., 1999; Quinn *et al*., 2007). When and how the resistances are generated has no effect on this estimation, which can be calculated in some of the antimicrobials, bacteriostatic or bactericidal. Thus, it has been estimated for macrolides, fluoroquinolones, β-lactams, linezolid, vancomycin and daptomycin (Zhao & Drlica, 2008). Some authors suggested the possibility that MPC could be estimated as a multiple of MIC but the low and

poor correlation between both values makes this estimation rather inaccurate (Sindelar *et al*., 2000; Marcusson *et al*., 2005).

Pharmacodynamic considerations

An important issue to have into account is that all the parameters described previously are determined *in vitro*, under standard and controlled conditions and not in the environment where the bacteria would grow *in vivo* such as milk, blood, intra or extracellular fluid, urine or surrounded by exudates or pus and with different Ph and oxygen conditions or even with another bacterial burden from which the PD parameters are derived. Furthermore, the *in vitro* determination of the PD parameters has not have into account the post antibiotic effect (PAE) or the post antibiotic leukocyte enhancement (PALE) that can be seen *in vivo* and can underestimate the antimicrobial effect.

The PAE describes the suppression of the growth of the bacteria after the removal of the drug of the site of action. The PAE effect is related to the drug, its concentration, the duration of the exposure and the microorganism. The PALE can be described as the increased susceptibility to phagocytosis shown by bacteria after the exposition to an antimicrobial. Antimicrobials which produce a great PAE effect tend to produce the greatest PALE.

1.4. PK-PD parameters

Generally, the settlement of a dosage regimen in new drugs has been based on linking data coming sometimes separately from PK or PD studies. Thus, the three main ways of choosing the most appropriate antimicrobial regimen are the followings: dose titration, PK-PD integration and PK-PD modelling.

Dose titration studies: This kind of studies has been historically used to establish doses and posology regimes in animals and humans. Healthy or

experimentally infected animals are randomly placed in one of the treatment groups and the outcomes are compared using statistical analysis. In antimicrobial drugs the outcome could be either the clinical recovery or the pathogen eradication. This type of studies has a lack of information on PK data on plasma and other tissues and consequently it is difficult to link them with the PD parameters (Toutain & Lees, 2004).

PK-PD integration: In these studies, *in vitro* PD determinations (usually MIC) are linked to one or more PK parameters (C_{max}, AUC and time above the MIC) coming from a separate PK study, in order to choose the most appropriate treatment that achieves the breakpoint value that will ensure the efficacy of the treatment (figure 4). PK-PD values and their breakpoints are explained largely afterwards. An example of this kind is the study performed by Aliabadi & Lees (2002) where they integrated the marbofloxacin PK data obtained in calf serum, exudates and transudate with the MIC of *Mannheimia haemolytica* calculated separately. This methodology has gained a greater importance lately for being an alternative less expensive and more effective than the dose-titration studies. Besides, current concerns on animal welfare do support PK-PD integration in front of dose titration studies.

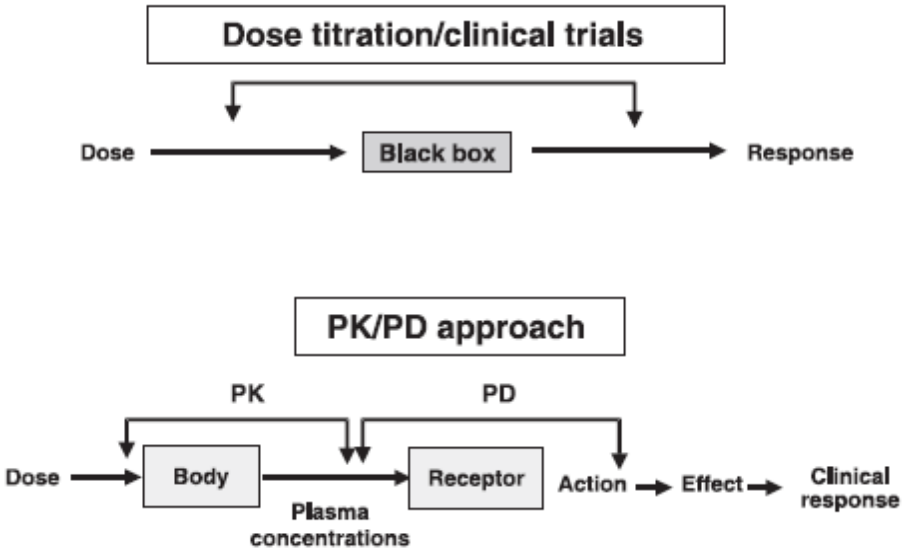


Figure 4. Schematic representation of dose titration and PK-PD studies (Toutain & Lees, 2004).

PK-PD modelling: PK-PD modelling is also, as PK-PD integration, an alternative cheaper and more effective than dose-titration studies. Modelling differentiates from integration in the way that modelling is carried out *in silico* using data coming from the PK analysis of a single dose and integration brings together PK and PD data coming from different (or the same) studies. Modelling requires having into consideration three stages. First, generating a PK model which is generally the traditional PK approach where the compartmental model and parameters are determined. Second, describe the link between the plasma and the biophase. And finally, the last stage, that relates the concentration with the effect (Holford & Sheiner, 1981).

A special type of PK-PD modelling is the one based in population PK studies. As commented earlier, population PK studies take into account the variability of individuals in a target population. On the other hand, bacteria show a non-normal MIC distribution. Thus, those data may be brought together in PK-PD analysis using Monte Carlo simulations (MCS). MCS consists in using computer software to increase the size of the targeted population to provide predictions on the achievement of the therapeutic thresholds. Therefore, MCS can incorporate the PK parameters variability and the MIC distribution of the selected bacterial population (Bonate, 2001; Frei *et al.*, 2008). Two of the estimates used in MCS are the probability of target attainment (PTA) and the cumulative fraction of response (CFR). The PTA describes the probability to achieve a certain threshold (in this case a PK-PD threshold) at a certain MIC. It may also be found as target attainment rate (TAR). The other term, the CFR, is the predicted probability of target attainment for a given antimicrobial dose and a specific bug population (Mouton *et al.*, 2005).

In conclusion, PK-PD information is very useful when it comes to the development of new antimicrobials, the more specific selection of appropriate antimicrobials from formularies, the design of optimal dosage strategies and the reduction of the selection of antimicrobial resistance (Gunderson *et al.*, 2001).

1.4.1. Clinical efficacy: Optimization for dosage regimens

The PK-PD parameters which have been mostly investigated, and for which the most robust information is currently available, are the ratio between the area under the curve with the MIC (AUC/MIC), the ratio between the maximum concentration and the MIC (C_{max}/MIC) and the time during the concentration is above a defined threshold, in this particular case, the MIC ($T_{>MIC}$) (Hyatt *et al*., 1995).

AUC/MIC usually refers to the area under the curve in a steady-state condition in a 24 h period of time. However, other time-periods can be used as long as it is indicate by adding a subscript (Mouton *et al.,* 2005). The right units for this index is hours, but sometimes can be found written without dimension (Toutain *et al.,* 2007). The relationship between efficacy and the ratio AUC/MIC has been well demonstrated for antibacterials in several studies in animals and humans. Thus, Legget *et al*. (1989) carried out a study in a mouse Gram-negative where the mortality was reduced with fluoroquinolones almost to 0% when the ratio AUC/MIC was above 100. Another study showed a relationship between the ratio and the bacteriological cure in ill people treated with the fluoroquinolone ciprofloxacin. In this latter study the Gram-negative bacteria was not isolated in almost 80 % of the patients when the AUC/MIC ratio was above 125 (Schentag, 2000). In veterinary medicine, the steady state is seldom reached as most of the drugs are designed to do their effect in a few applications of the drug. That is even more complicated when the drug has to be administered in livestock where the animals are staying in pens and the handling and treatment of the ill animals is not as easy as in pets. In those cases where the steady state is not reached, the $AUC_{0-\infty}$ should be used as an equivalent of the AUC mentioned before when the dose interval is every 24 hours. If the dose interval is longer it can be used the AUC of the interval covered by the drug activity or the time segment of interest (Papich, 2014; Toutain *et al.,* 2007).

The $T_{>MIC}$ defines the percentage of time during which the drug concentration exceeds the MIC at steady-state in a 24h period (Mouton *et al*., 2005). This

parameter correlates especially well with efficacy in β-lactam antibacterial drugs. Thus, a study conducted for Craig & Andes (1996) in people with otitis media treated with amoxicillin showed that the bacterial cure rose from 40 to 80 % when the T above MIC increased from 10 to 100 %. However, it is still unclear how much above the MIC the antimicrobial concentration should be kept. As a general rule, it is conceived that the concentration has to be maintained 1 to 5-fold the MIC during 40 or 100% of a 24 h period at steady state (Mckellar *et al.,* 2004). In order to optimize the treatment based on T above the MIC it should be recommended to use repeated doses in short intervals to avoid the plasma concentration falling under the MIC. The continuous infusion administration of β-lactam would provide a very precise method for keeping the drug plasma concentration above the MIC although it is not a frequent practice in veterinary medicine except for intensive care or during anaesthesia (Sarasola & McKellar, 1993).

The relationship with the pharmacological peak concentration and MIC is generally expressed as C_{max}/MIC. This term has no units as expresses a ratio. Some other terminology can be found, such as inhibitory quotient (IC) or inhibitory ratio (IR), although the term C_{max}/MIC is more extended and easy to understand (Papich, 2014). A clear relationship has also been demonstrated between C_{max} and a favorable clinical outcome in Gram-negative infections treated with aminoglycoside and fluoroquinolones antimicrobials. Regarding to the aminoglycosides treatment in humans, differences in clinical response were seen if the ratio C_{max}/MIC was 2 or 12 having a favorable clinical response in about 50% or 90 % of the patients respectively (Moore *et al.,* 1987). On the other hand, other studies confirm the suitability of using this ratio in fluoroquinolone treatments to predict the positive clinical outcome. Thus, Drusano *et al.* (1993) showed the importance of this ratio in a neutropenic rat model infected with *Pseudomonas sepsis* and treated with moxifloxacin. This model linked survivorship with the C_{max}/MIC ratio especially when high ratios were achieved (10 to 20). Some other research in cattle respiratory disease model carried by Sarasola *et al*. (2002) confirmed that the maximum therapeutic benefits are obtained with the administration of high doses of danofloxacin are

preferred instead of continuous infusions when treating *Manhemia haemolityca* infections.

According of all expressed before, antimicrobial drugs may be classified as concentration-dependent where increasing concentrations at the locus of infection improve bacterial kill, or time-dependent where exceeding the MIC for a prolonged percentage of the inter-dosing interval correlates with improved efficacy. For the latter group, increasing the absolute concentration obtained above a threshold does not improve efficacy. A third group of antimicrobials can be described, co-dependent antimicrobials, whose effect on bacteria depends as much on the exposure time as on the concentration reached (table 1). The indexes that best correlates with clinical efficacy for each group are C_{max}/MIC for concentration-dependent compounds, AUC/MIC for co-dependent antimicrobials and T above MIC for time-dependent drugs. The PK-PD relationship for each group of antimicrobial drugs is "bug and drug" specific, although ratios of 100-125 for AUC/MIC and 8-10 for C_{max}/MIC have been recommended to achieve high efficacy for co-dependent and concentration-dependent antimicrobial drugs, respectively, and exceeding MIC by 1-5 multiples for between 40 and 100% of the inter-dosing interval is appropriate for most time-dependent agents (McKellar *et al.,* 2004).

Concentration-dependent	Time-dependent	Co-dependent
Aminoglycosides	Beta-lactams	Beta-lactams*
Fluoroquinolones	Macrolides (except azithromycin)	Fluoroquinolones**
Metronidazole (vs. Anaerobes)	Clindamycin	Glycopeptides
	Vancomycin	

Table 1. General classification of antimicrobial drugs according to the information available on concentration or time-dependent killing activity (*In relation to reduction in resistance selection pressure;**Some with anaerobic activity). McKellar *et al*., 2004.

1.4.2. Preventing antimicrobial resistance

Recently, some authors suggested the possibility that PK parameters linked to the MPC could foresee the prevention of appearance of resistances. Dr. Baquero was the first author to introduce a new concept in therapeutics to prevent the appearance of resistant mutants, the mutant selection window (Baquero, 1990; Baquero & Negri, 1997). The mutant selection window (MSW) postulates that any antimicrobial concentration that falls within a given range during the antibacterial therapy, will amplify the mutant subpopulation found in any given bacterial population. The lower boundary of the MSW is the lowest concentration that inhibits the growth of the 99% of susceptible cells (MIC_{99}). The upper boundary of the MSW is the MPC, above which any growth is allowed and no mutants are not selected in large microbial burdens (>10^9 CFU) (Drlica & Zhao, 2007). This theory has been proved in gram-negative and gram-positive bacteria in a tissue cage infection model in rabbits. Ni *et al.* (2014) showed an increase of recovered mutants of *Escherichia coli* when levofloxacin concentration fell within the MSW. Another study compared various doses of levofloxacin and found that a mutant subpopulation of *Staphylococcus aureus* is enriched when concentrations of the antimicrobial are within the boundaries of the MSW (Cui *et al*., 2006).

The MSW hypothesis is easy to understand. Thus, the longer the time the drug concentration is within the boundaries, the higher the probability of appearance of resistances. However, the thresholds or parameters derived from it are not clear enough. Several indicators have been proposed to have into account when settling an antimicrobial regimen to avoid the selection of resistant mutants.

The first indicator which links PK and PD data is straightforward. The time the antimicrobial concentrations were between the MSW boundaries during the dosing interval (T_{MSW}) was linked to the enrichment of mutants in *in vitro* and *in vivo* studies (Almeida *et al.*, 2007; Firsov *et al.,* 2003; Ferran *et al.,* 2009). However, information about a threshold brought by different authors is

confusing. Firsov *et al.* (2003) suggested that drug concentrations should fall in the MSW more than 20 % of the posology regimen in an *in vitro* study with four fluoroquinolones to enrich the resistant subpopulation, whereas Ferran *et al*. (2009) found that a T_{MSW} less than 30 % of the posology regimen would predict the prevention of mutant selection. Another research in a rabbit pneumonia model infected with pneumococci showed a range of T_{MSW} between 2 and 25 % for levofloxacin and between 72.5 and 93.5 % for moxifloxacin to prevent the growth of mutants (Croisier *et al*., 2004). Even some other authors could not find a relationship between the T_{MSW} and the enrichment of the mutant subpopulation in a rat lung infection with *Klebsiella pneumonia* and treated with marbofloxacin (Kesteman *et al*., 2009). It was suggested that this data variability and the incapacity of foresee the appearance of resistances by the T_{MSW} could be explained by the fact that this parameter does not discriminate between the concentrations at the top or the bottom of the MSW and that could generate confusion since it is not the same when time outside the MSW are above the upper boundary, MPC, or under the lower boundary, MIC (Firsov *et al*., 2008).

Another proposed indicator that linked PK and PD data on the prevention of bacterial resistances appearance is the time above the MPC ($T_{>MPC}$) which is the minimal time that concentrations should be above the MPC to restrict the growth of the single step mutants and is expressed as a percentage over time or over the treatment period. Contradictory information can be found in the literature about this index. Whilst some studies support the idea that $T_{>MPC}$ is linked to the reduction of susceptibility, Cui *et al*. (2006) found correlation between the threshold $T_{>MPC}$ of at least 20% of the treatment time of levofloxacin against *Staphylococcus aureus* in a rabbit model, some other studies didn't found any link to the appearance of resistances in *Streptococcus pneumoniae*, *Escherichia coli* or *Staphylococcus aureus* (Homma *et al.,* 2007; Olofsson *et al.*, 2006). Therefore, more studies involving the prediction of this index on resistances are needed in order to clarify whether it could be a good predictor or not and the threshold values that it should have in case its value was demonstrated.

A third recently new indicator, crossed the previous two, T_{MSW} and $T_{>MPC}$, in a ratio, the $T_{>MPC}$ / $T_{MSW.}$ This index was first described in the paper of Kesteman *et al*. (2009) in order to overcome the difficulties found by the other two indexes to predict the enrichment of resistant mutants and puts together two different processes, the time in which all bacteria is eliminated ($T_{>MPC}$) and the time where the mutants are enriched and selected. One of the advantages that Kesteman's paper pointed of this ratio is that allows us to compare fractionated and single-dose administrations. It is worth noticing that for fractionated administrations the ratio over a 24 hour period will be the same as the total duration of the treatment. However, this is a very new parameter and cut-off values for this ratio are not known. So it would need to be further studied.

Some authors have suggested the use of the PK-PD MPC-based parameters instead of the PK-PD MIC-based parameters (AUC/MPC and C_{max}/MPC (Homma *et al*., 2007)) to find an appropriate index to predict the reduction of the susceptibility in fluoroquinolones. Nevertheless, a recent research that compared the suitability of PK-PD indices in levofloxacin resistant strains of *Staphylococcus aureus* pointed that the use of AUC/MPC should be preferred instead of the C_{max}/MPC since the first takes into account the posology regimen and the second does not (Liang *et al*., 2011).

AUC/MPC, as commented previously, is an index that derivates from the PK-PD efficacy index AUC/MIC and it is expressed in hours. As early indicated, AUC/MPC seems to be a better predictor as other PK-PD indicators. Despite that several studies have tried to find a threshold for restricting the appearance of less-susceptible sub-populations this cut-off value is not clear yet. While some authors pointed to an AUC/MPC threshold value around 70 h for fluoroquinolones for preventing the enrichment of mutant sub-populations in *Staphylococcus aureus in vitro* (Firsov *et al.,* 2003; Firsov *et al.,* 2004) some others indicated that an AUC/MPC value that felt between 20 to 25 h would prevent the appearance of resistances in the treatment with fluoroquinolones of *Staphylococcus aureus* and gram-negative bacteria (*Streptococcus*

pneumoniae and *Escherichia coli)* in both, *in vitro* and in a rabbit model (Liang *et al*., 2011; Ni *et al*., 2014; Cui *et al*., 2006).

In conclusion, researchers are doing a big effort to find new tools to avoid the selection of resistances whilst improving the treatments with the existent antimicrobials. Whilst PK-PD predictors of efficacy seem very consistent, there is still a lot of work to do in those PK-PD predictors that attempt to restrict the appearance of resistances under control.

1.5. MIC breakpoints

The success or failure on antimicrobial therapy in infections caused by bacteria is usually predicted by testing the infectious agent susceptibility in *vitro* and categorizing the bacteria into three groups according to the MIC breakpoints: susceptible (S), indeterminate or intermediate (I) and resistant (R). In other words, S would mean the antimicrobial activity would be associated with a likelihood of therapeutic success, I where the result of the therapeutic treatment would be uncertain and R where a high probability of treatment failure exists (International Organization for Standards, 2006). Another set of definitions is provided by the Clinical and Laboratory Standards Institute (CLSI, 2007) where S, I and R categories are related to the achievable concentrations with normal dosage schedules at the site of action. MIC breakpoints can be estimated either directly, MIC determination on agar or broth, or indirectly, on disc diffusion techniques and then converted to inhibition zone diameters. In addition, MIC breakpoints do not have into account the appearance or development of resistant strains.

In the existent literature it can be found a variety of uses for the term breakpoint (Wikler & Ambrose, 2005). The first use is the “wild-type breakpoint” (also referred as microbiological breakpoint) and refers to the MIC for any given antimicrobial that separates wild-type population of bugs from those who have acquired resistance mechanisms. This breakpoint derivates from MIC data

collected after from moderate to large number of in vitro susceptibility tests. A second use of the word is the “clinical breakpoint”. This breakpoint separates strains according to the likelihood of success or failure of the treatment. In their simplest way, these values came from clinical studies that compared outcomes with different MICs of the infectious agent. A third use involves the “PK-PD breakpoints”. These breakpoints are derived from the knowledge of a PD parameter and their reflection in the *in vivo* positive outcome. Since additional methodologies are currently used to evaluate PK-PD relationships of antimicrobials being PK-PD models and Monte Carlo simulations two of these techniques, they can be used to improve interpretive susceptibility criteria based on PK-PD principles (PK-PD breakpoints). People who advocate for PK-PD simulations claim that the resulting breakpoints reflect more the real antimicrobial effectiveness in the population than the traditional techniques. Also this approach improves the detection of drug resistance and makes easier the design of antimicrobial regimes (White, 2001). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has also adopted PK-PD simulations as a key to set breakpoints either for old and new antimicrobials (Kahlmeter *et al*., 2003). Thus, in PK-PD simulations the breakpoints are set at the highest MIC value that the PTA is ≥90% as this is the recognized target attainment cut-off used by the American counterpart of the EUCAST, the CLSI, when defining MIC breakpoints (Maglio *et al.,* 2005). Finally, some authors suggested that the terms epidemiological cut-off, wild-type cut-off or PK-PD cut-off value should be preferred in front of the term “breakpoint” in any of their uses and leave the use of the term breakpoint be reserved for the final value for the clinical laboratory and to guide the therapy (Kahlmeter *et al*., 2003; Turnidge & Paterson, 2007).

MIC breakpoints are usually defined mainly by the CLSI (formerly known as NCCLS) and/or for any of the several actives national committees (BSAC in the UK, CASFM in France, CRG in the Netherlands, DIN in Germany, NWGA in Norway and SRGA in Sweden) that conform the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These two groups publish guidelines about the required data and how this data is applied to breakpoints setting (EUCAST; CLSI, 2012). Some other regulatory agencies such as the

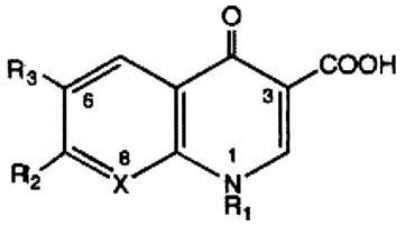
Food and Drug Administration (FDA, USA) or the European Medicines Evaluation Agency may define breakpoints temporarily or permanently.

2. QUINOLONES AND FLUOROQUINOLONES

2.1. Introduction

Origin and chemistry

Quinolones are a synthetic antimicrobial group first discovered in 1962 when Leicester and colleagues accidentally discovered the nalidixic acid (figure 5) as a by-product of the antimalarial compound chloroquine (Lesher *et al*., 1962). Because of its limitations in absorption and distribution, nalidixic acid only reached therapeutic concentrations in urine, and its restricted antibacterial spectrum to the Gram-negative bacteria, nalidixic acid action was effective solely against urinary tract infections.



X = N; naphthyridone

X = CH ; quinolone ;

Nalidixic acid ; $R_1 = CH_2CH_3$;
R_2 = pyridyl
R_8 = H
X = N

Figure 5. Nalidixic acid. (Martinez *et al*., 2006)

It was not until the 80s when the real advance in the development of the quinolones was produced. First, the addition of a fluorine molecule at the 6-position of the basic quinolone structure which increased DNA gyrase inhibitory activity, facilitated penetration into the bacterial cell and extended the quinolone

effect against Gram-positive bacteria. Second, the addition of a piperazine group at C-7 enhanced the antibacterial activity against aerobic Gram-negative bacteria, increased the activity against both staphylococci and Pseudomonas species and improved the tissue distribution (Andriole, 2005). These modifications lead to the development of the first wide spectrum fluoroquinolone (FQ), norfloxacin, first approved for use in the USA in the mid 80s. Since then, other FQ have been developed and are available for antimicrobial treatment.

Some authors suggest that FQ may be classified into different categories according to their activity and pharmacokinetics. However, the information found in the literature did not enclose the groups well (Ball, 2000; Oliphant & Green, 2002; King *et al*., 2000; Lee & Sanatani, 1999; Van Bambeke *et al*., 2005). According to Martinez *et al.* in a 2006 review, FQ can be divided into four generations where most of the veterinary used FQ can be found in the second or third generation group. The first generation group comprises the early quinolones nalidixic acid, oxolinic acid, pipemidic acid and cinoxacin. These compounds achieved low serum and tissue levels due to their poor oral bioavailability and limited distribution. Besides, the first generation molecules have a narrow gram-negative coverage. The second generation comes from the norfloxacin synthesis (first FQ) and includes as a sample, norfloxacin, ciprofloxacin, enrofloxacin, danofloxacin, difloxacin, sarafloxacin and enoxacin. This group shows increased antibacterial activity against Enterobacteriaceae and other Gram-negatives. Furthermore, improved oral bioavailability and tissue distribution are associated with this category. The third generation drugs kept the favorable pharmacokinetic characteristics but expanded the FQ activity to Gram-positive bacteria (*Streptococcus pneumonia*) and atypical pathogens such as *Mycoplasma pneumoniae* and *Clamydophila pneumoniae*. However, this group is less active than ciprofloxacin against Pseudomonas species. Some examples of this group are levofloxacin, marbofloxacin, grepafloxacin, moxifloxacin and sparfloxacin. And last, the fourth generation group, which has significant antimicrobial activity against anaerobes and Pseudomonas (comparable to ciprofloxacin) while keeping the same activity than the third generation fluoroquinolones against Gram-negative and Gram-positive. This group includes trovafloxacin, gatifloxacin, moxifloxacin, gemifloxacin and

sitafloxacin. In adition, Somasundaram & Manivannan (2013) proposed a fifth category with only one antimicrobial, delafloxacin which has activity against Gram-negative and Gram-positive bacteria, showing activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and against multidrug-resistant isolates (Remy *et al*., 2012).

Mechanism of action and antibacterial activity

Since their discovery, the activity spectrum of these drugs has been developed considerably, leading to a wide spectrum of action against gram-negative bacteria, mycoplasma and some gram positive bacteria. FQ affect the DNA supercoiling by inhibit an enzyme found in all bacteria, the DNA gyrase, that plays a vital role in DNA packing, replication and transcription. Furthermore, the FQ have a secondary target, the topoisomerase IV that acts in the ATP dependent relaxation of the DNA (Martinez *et al*., 2006).

In addition to their antimicrobial activity, a FQ post-antibiotic effect (PAE) that decrease or affects the growth of the bacteria after the drug exposition is exhibit in some bacteria-drug combinations. According to Athama *et al.* (2004) the *in vitro* PAE effect of fluoroquinolones on *Bacillus anthracis* ranged from 2 to 5 hours. Furthermore, the duration of the PAE effect tend to be longer *in vivo* than *in vitro*.

2.2. Pharmacokinetics and toxicity

Regarding to the general pharmacokinetic properties of the FQ:

Absorption

Fluoroquinolones usually have a good and quick oral absorption showing variability depending on the agent administered and the animal species when administered to monogastric species and pre-ruminants. Nevertheless, therapeutic concentrations may not be reached when FQ are administered orally in ruminants. The administration of oral FQ with food does not seem to affect the bioavailability or the absorption rate further than delaying the peak concentration in plasma. However, if FQ are administered together with foods containing divalent cations, a decrease in the FQ bioavailability will occur. An example of the variability of the oral bioavailability is shown by enrofloxacin in different species such as dogs (91%), chicken (101%), turkey (61%), cattle (8%) and horse (60%) (Aminimanizani *et al*., 2001; Bergogne-Berezin, 2002; Wright *et al*., 2000). On the other hand**,** parenteral bioavailability (intramuscular or subcutaneous administration) is complete or nearly complete in most of the quinolones, even in ruminants.

Distribution

The activity and the distribution of the drug from the plasma to the tissues are dependent on the plasma concentration of the drug that is not bound to proteins. This binding to proteins is different according to each compound. For instance, in humans, over the 90 % of the nalidixic acid binds to plasma proteins, ciprofloxacin binds to proteins around 20 % and norfloxacin is bound between 10 and 15 % to plasma proteins (Brown, 1996). However, most of the FQ showed a lack of protein affinity (<50%) and they bind predominantly on albumina (Bergogne-Berezin, 2002).

FQ usually distribute well and have larger volumes of distribution than the total body water (Bregante *et al*., 1999) indicating that they are concentrating mainly

in tissues and showing tissue/plasma concentration ratios higher than 1. Besides, it is important to highlight that FQ have the capacity to concentrate in fagocitic cells.

Metabolism and excretion

FQ metabolism and excretion occurs in the liver and in the kidney, respectively. However, the different FQ can use different pathways of elimination using mainly hepatic metabolism (difloxacin and perfloxacin), renal mechanisms (enrofloxacin, orbifloxacin, temafloxacin and lomefloxacin) or a combination of both, renal and hepatic mechanisms (marbofloxacin, danofloxacin, norfloxacin, ciprofloxacin and enoxacin) (Karablut & Drusano, 1993). The mechanisms that involve the hepatic metabolism include glucuronidation (cinafloxacin, grepafloxacin, sparfloxacin and moxifloxacin) and N-oxidation and desmethylation (levofloxacin and sparfloxacin). Sulfoxidation and acetylation are other possible metabolic pathways. Generally the CYP 450 system is involved in the metabolization (Bergogne-Berezin, 2002; Lode *et al*., 1990).

Enteropatic recirculation is possible as some fluoroquinolones may be eliminated through biliar or intestinal excretion. Renal excretion of the FQ involves glomerular filtration and active tubular secretion and the range of this elimination is different depending on the FQ.

Toxicity

The FQ are a group of antimicrobials relatively save. The therapeutic use may produce low or mild toxic effects generally related to the gastrointestinal system such as nausea, vomiting, diarrhoea. Furthermore, signs as photosensivity, central nervous system effects (seizures, insomnia, ataxia, dizziness, restlessness, headache, somnolence and tremors) and crystalluria (leading to obstructive uropathy) have been described when administered at higher doses.

Type of adverse event	Specific adverse event
Gastrointestinal	Nausea, vomiting, abdominal pain, diarrhoea, anorexia
Central Nervous System	Headache, dizziness, sleep disorder(s), mood changes confusion, delirium, psychosis, tremor, seizure
Hepatic	Transient rise in level of liver function enzymes, cholestatic jaundice, hepatitis, hepatic failure
Renal	Azotemia, crystalluria, hematuria, interstitial nephritis, nephropathy, renal failure
Dermatologic	Rash, pruritus, photosensivity, hemorrhagic bullae, leg pigmentation, urticaria
Musculoskeletal	Arthropathy, tendinitis, tendon rupture
Cardiovascular	Hypotension, tachycardia, QT interval prolongation
Others	Drug fever, chill serum-like reaction, anaphylactoid reaction, anaphylaxis, angioedema, bronchoespasm, vasculitis

Table 2. Most frequently reported adverse events associated with (FQ) antibacterials (Lipsky & Baker, 1999).

The FQ administration to growing animals can be related to the production of non-inflammatory erosive arthropathies, especially in weight-bearing joints in dogs (large breeds) and foals. In addition, ocular toxicity as retinal degeneration and subcapsular cataracts may be observed in cats after the administration of high doses of FQ (Papich & Riviere, 2009). A summary of the frequent adverse events related to FQ therapy in humans is shown in table 2 reproduced from Lipsky & Baker (1999).

Specie	Type of infection	Formulations
Ruminants	Respiratory and enteric	Injectable and bolus
Swine	Respiratory, enteric, mastitis/metritis	Injectable, oral suspension and medicated feed.
Dogs and cats	Dermic/wounds, urinary and respiratory.	Oral (tablets) and injectable
Horses	Respiratory, enteric, metritis and joint	Injectable and oral
Poultry	Enteric and respiratory	Oral (water)
Fish	Septicemia and skin ulcers	Oral (feed) and water (bath treatments)
Rabbits	Enteric and respiratory	Injectable and oral (water)

Table 3. Therapeutic uses and presentation form of FQ by species. (FDA, 2014; EMA, 2010)

2.3. Fluoroquinolones in veterinary medicine

As well as in human medicine, FQ have a wide spread use in veterinary medicine to treat different types of infections, especially those caused by Gram-negatives and some Gram-positives, in all kinds of animals, livestock, fish and pets. However, it should be avoided the use of this drugs in young animals, especially dogs, because FQ may induce arthropathy in these animals (Maslanka *et al*., 2004). The first FQ approved for veterinary medicine was enrofloxacin in 1988 (Vancutsem *et al*., 1990). Since then, some other FQ have been approved for this use and have been also withdrawn of it. A summary of the therapeutic indications and the formulations that you may find with FQ are listed in table 3.

		Livestock	Poultry	Pets	Aquiculture	Other species
USA[a]						
	Danofloxacin	Only cattle				
	Difloxacin			Only dogs		
	Enrofloxacin	Cattle and swine		Cats and dogs		
	Marbofloxacin			Cats and dogs		
	Orbifloxacin			Cats and dogs		
	Pradofloxacin			Only cats		
	Ciprofloxacin	Not approved for veterinary use yet is used for dogs and cats				
	Sarafloxacin		Restricted uses in poultry.			
EU[b, c]						
	Pradofloxacin			Cats and dogs		
	Difloxacin	Cattle	Chicken and turkey	Dogs		
	Enrofloxacin	Cattle and swine	Chicken and turkey	Dogs and cats		Rabbits
	Marbofloxacin	Cattle and swine		Dogs and cats		
	Danofloxacin	Cattle and swine				
	Flumequine[d]	Cattle (non-ruminants), lamb and swine	Chicken, turkey, hens	Dogs	Fish in general	Horse, rabbits and fur animals
	Orbifloxacin			Dogs		
	Oxolinic acid[d]	Cattle and swine	Poultry in general		Trout	Calves

Table 4. List of FQ approved within the US and EU for veterinary use in 2014. The compounds approved within the UE may vary from one country to another having differences in FQ and species registered. [a] Food and Drug Administration (FDA). [b] European Medicines Agency (EMA), [c] Agencia Española de Medicamentos y Productos Sanitarios (AEMPS).[d] Flumequine and oxolinic acid are actually quinolones.

Different approved FQ in veterinary medicine can be found in different countries or territories according to their regulations. USA is very restrictive about the FQ use in food animals to avoid food-borne resistances although it is more permissive in pets. On the other hand, the FQ use to treat infections in animals is wider in the EU than in USA and extends their use to food producing animals, horses, pets and fish. Nevertheless, the EU is continuously monitoring their veterinary use and supporting the idea that FQ should be used with care in animals. FQ used for veterinary species within the USA and EU are listed in table 4 and their chemical structure is depicted in figure 6.

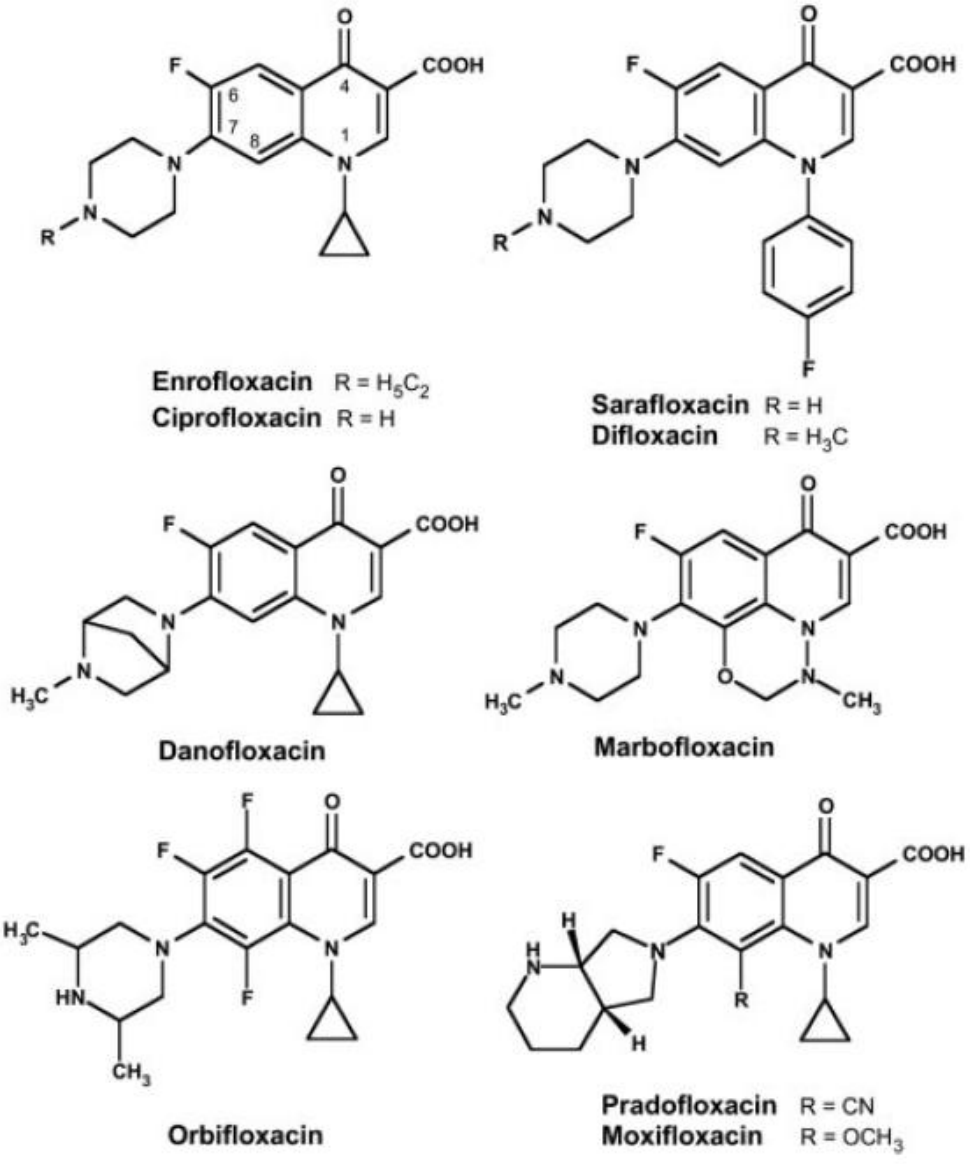


Figure 6. Chemical structures of different fluoroquinolones approved for use in animals (Wetzstein, 2005).

2.3.1 Marbofloxacin PK and use in swine medicine

Marbofloxacin (MB) (figure 7) is a third generation fluoroquinolone widely used in veterinary medicine. The main chemical particularity of MB is the oxadiazine cycle that confers a long elimination half life and excellent bioavailability to the molecule.

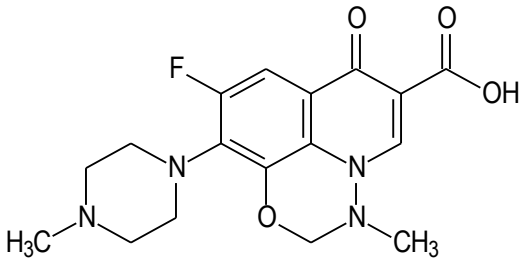


Figure 7. Chemical structure of Marbofloxacin. $C_{17}H_{19}FN_4O_4$. 9-fluoro-2, 3-dihydro-3-methyl-10 (4-methyl-1-piperazinyl)-7-oxo-7H-pyrido (3, 2, 1-ij) (4, 1, 2) benzoxadiazine-6-carboxylic acid (Kindly provided by Vétoquinol International).

This molecule is an organic acid with some of the characteristics commented previously for FQ:

- Very good absorption and excellent bioavailability regardless the administration route. Absolute bioavailability by intramuscular route in swine was between 90 and 100% in weaners and fatteners (Ding *et al*., 2010; Schneider *et al*., 2014) independently of the dose and drug concentration. Ding and colleagues (2010) tested the pharmacokinetics of a MB at a dose of 2.5 mg/kg bw of MB in weaners using a 2% solution. On the other hand, the study of Schneider *et al*. (2014) focused on the PK of MB at different doses (4, 8 and 16 mg/kg bw) using a 16 % solution of MB. MB bioavailability was nearly 100 % in all the species registered. The lowest bioavailability was found after its oral administration MB in horse (62.44%) (Anonymous, 1997).

- Good tissue penetration. Its volume of distribution exceeds the body water volume and the degree of binding to plasma proteins is low (Sidhu *et al*., 2010). The values of the volumes of distribution in the MB studies of Ding *et al.* (2010) and Schneider *et al*. (2014) are1.3±0.14 and 1.58±0.26 L/kg respectively. Besides, the tissue/plasma concentration ratio is greater than 1 in most of the tissues which suggests a certain degree of tissue accumulation.

- MB has a long elimination half-life. MB is eliminated as an active molecule mainly in the urine (60%) although one third is eliminated via faeces. Metabolism and biotransformation in the liver is low but it leads to the formation of two metabolites (marbofloxacin N-oxide and demethyl-marbofloxacin). Different values of swine clearance can be found in the literature depending on the study: 0.12-0.2 L/h/kg for pregnant and lactating sows (Petracca *et al*., 1993), 0.12 L/h/kg for 10 week-old pigs (Ding *et al*., 2010) and 0.092 L/h/kg and 0.079 L/h/kg for weaners (12 weeks old) and fatteners (16 weeks old) respectively (Schneider *et al*., 2014). These differences would be explained by the fact that pregnant animals have an increased clearance of those drugs that are eliminated mainly in the urine (Dvorchik, 1982). Other differences in total clearance can be explained due to the physiological changes that age could produce in kidney and liver (Schneider *et al*., 2014).

A graphical representation of the concentration time profile can be seen in figure 8 and a summary of the main MB pharmacokinetic values in different species can be seen in table 5.

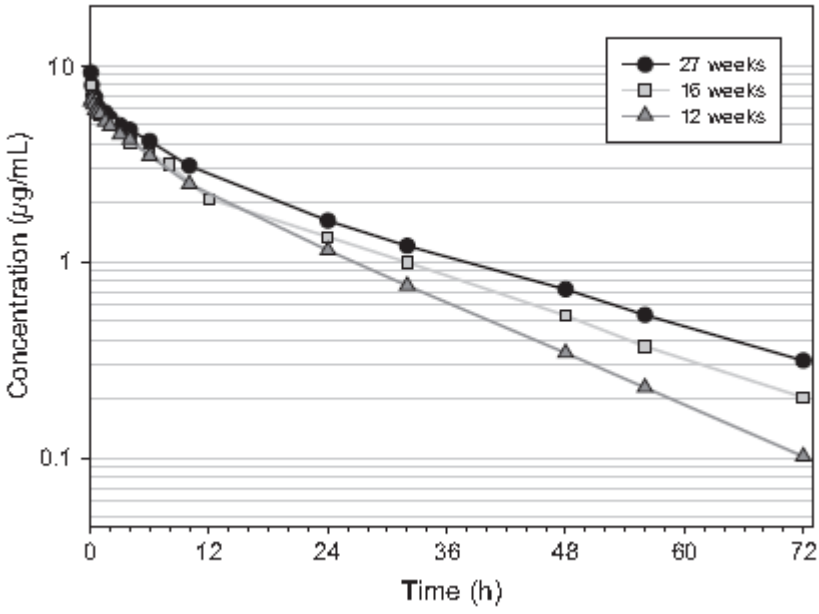


Figure 8. Plasma concentration vs. time profiles of marbofloxacin after a single intravenous administration dose of 8 mg/kg in pigs of different ages (Schneider *et al*., 2014).

		Cat	Dog	Swine	Cattle (*Calve*)	Sheep [b]	Horse[b]
F%		97.6 (SC) 84.6 (PO)	98.7 (SC) 99.77 (PO)	100(IM)	100(*100*)(IM) 100(*91.81*)(SC) *(Nd)(PO)*	100 (IM)	97.59 (SC) 62.44 (PO)
Vss (L/kg)[a]		1.48	1.36	1.77	1.16 (1.35)	1.49	1.48
T1/2 (h)		10.28 (IV) 13.12 (SC) 8.54 (PO)	12.40 (IV) 13.00 (SC) 9.77 (PO)	8.24 (IV) 9.48 (IM)	5.72 (*7.84*)(IV) 7.73 (*9.12*)(IM) 5.49 (*9.05*)(SC) (*23.3*)PO	2.02 (IV) 2.10 (IM)	7.56 (IV) 10.41 (SC) 8.78 (PO)
Ratio Tissue/Plasma Concentration [c]	muscle	1.98	1.6	1.7	1.3 (*1.33*)	ND	ND
	liver	3.22	2.5	1.9	2.1 (*2.09*)	ND	ND
	kidney	2.23	2.3	3.8	4.5 (*4.49*)	ND	ND
	lungs	1.45	1.5	1.8	1.3 (*1.32*)	ND	ND
	fat	0.5	0.5	ND	ND (*ND*)	ND	ND
	skin	1.72	1.6	1	ND (*ND*)	ND	ND

Table 5. Main pharmacokinetic parameters of different administrations in different species following the administration of 2 mg/kg of marbofloxacin (Marbocyl®). [a] After the IV administration. [b] MB is not registered for sheep or horse in any UE country (EMA, 2010).[c] The ratio was determined: 2 hours after a single oral dose of 2 mg/kg in cat (except skin, after 5 days of oral treatment at 2mg/kg), 48 h after the last dose of 4 mg/kg/d by oral route for 7 days in dog (except skin that was determined 24 hours after the last dose of 2mg/kg/day for 13 weeks), 4 hours after the administration of 2mg/kg in pigs and after 4 and 2 hours of a single IM administration of 2 mg/kg in cattle and calve respectively. Anonymous, 1997.

MB possesses a broad spectrum of activity against mycoplasma, most Gram-negative, some Gram-positive bacteria and some intracellular pathogens such as *Brucella* and *Chlamydia* species, but with limited or no activity against anaerobes (Hannan *et al*., 1989; Spreng *et al*., 1995; Appelbaum & Hunter,

2000). This spectrum of activity includes some of the swine respiratory pathogens of the porcine respiratory disease complex (PRDC), such as *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*. Furthermore, MB could be very useful to treat the mastitis-metritis-agalactia (MMA syndrome where the common etiologic agents involved are enterobacteria (mainly *Escherichia coli*) against which fluoroquinolones have shown their effectiveness.

Besides swine MB is also registered for cattle, dogs and cats in some of the EU countries as well as in Spain. Some of the indications for these species are summarized below:

- Dog: Its applications are against skin, urinary and respiratory system, otic or soft tissues infections caused by MB susceptible bacteria. In addition, it can be used as a prophylactic drug after a surgery to prevent common infections caused by *Staphylococcus intermedius*, *Escherichia coli* and Pseudomonas

- Cat: MB may be administered to treat infected wounds or abscess, upper respiratory infections or as a prophylactic after a surgery.

- Cattle: The main uses in cattle are to treat respiratory infections (caused by *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis* and *Pasteurella multocida*) and to cope with acute mastitis caused by *Escherichia coli* marbofloxacin susceptible. Moreover, in calves MB is used to treat gastrointestinal infections of *Escherichia coli*.

3. USE OF MARBOFLOXACIN TO TREAT RESPIRATORY DISEASE IN PIGS

3.1. Respiratory disease in pigs.

The PRDC is clinically characterized by dyspnea, coughing, acute depression, anorexia, fever, and nasal discharge, specially affecting growing to finishing pigs (Dee, 1996). This complex disease is most often due to the interaction of multiple factors. Both viral and bacterial organisms play a role, as well as the

environment and various management practices employed by producers. When in the right combination, these factors can sufficiently compromise the pig respiratory defense mechanisms, resulting in severe respiratory disease (Thacker, 2006). The most common viral pathogens associated with PRDC are porcine respiratory and reproductive syndrome virus (PRRSv), swine influenza virus (SIv), pseudorabies virus (PRv) and porcine respiratory coronavirus (PRCv). The most common bacterial pathogens associated with this complex include: *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Pasteurella multocida, Haemophilus parasuis, Streptococcus suis, Arcanobacterium pyogenes, Salmonella choleraesuis* and *Actinobacillus suis* (Christensen *et al*., 1999).

When bacterial infections in the respiratory tract occur, a treatment with an appropriate antimicrobial should be applied. The sensitivity test is the method to select the best drug for this treatment against a specific pathogen although it is not an infallible method.

Drug pharmacokinetics will help us to predict the drug concentration in blood or plasma. Nevertheless, the blood concentration of a drug doesn't allow us to predict the concentrations in other tissues that could be the target organ. Hence, sampling tissues is the best way to know how the drug distributes into the body. The coefficient of partition (P) is a ratio that can be found in the literature and relates the blood concentration at steady state and the tissue concentration of a given drug. Thus, this coefficient shows us how the drug is accumulated in a tissue and allows us to foresee the efficacy of an antimicrobial treatment when there is not more information available (Sanchez-Rubio & Sanchez, 1999). When calculating the drug concentration in an organ or tissue, it is usually minced and then the homogenate concentration is determined. Therefore P has to be taken as an indication and not as an absolute reference value since it cannot be specified in which part of the organ (lung) the drug is concentrated.

Knowing where the bacteria are located is critical to predict the success or failure of a treatment with antimicrobials. More specifically, in the case of respiratory diseases caused by bacteria, the bugs can be found free or inside the macrophages in the bronchial mucus and the alveoli (Fraile, 2013). Therefore, it would be very useful to know the antimicrobial concentration in bronchial secretions and inflammatory exudates to evaluate more specifically the antimicrobial therapy instead of using the blood levels of the drug (McKellar *et al*., 1999). To tackle this matter, some researches attempted to extrapolate or calculate the drug in the alveolar inflammatory exudate or lung tissue by using different approximations such as direct samples of bronchial secretions, tissue cages or microdialysis (McKellar *et al*., 1999; Aliabadi & Lees, 2002; Liu *et al*., 2005). Recent studies focused on the antimicrobial concentration in the pulmonary epithelial lining fluid (PELF) which seems to be the best way to predict the outcome of a treatment as bacteria are usually adhered to bronchial mucus and the alveoli. Thus, Giguère *et al*. (2011) determined the concentration of gamithromycin in plasma, PELF, bronchoalveolar cells and lung tissue in cattle and compared their pharmacokinetics. In another study, Villarino *et al*. (2013) showed that tulathromycin concentrations reached in the PELF are probably in the therapeutic concentration range despite the low plasma concentrations observed at the same time. Some other studies with fluoroquinolones in swine pointed that the ratio between free drug concentration of danofloxacin in PELF and plasma was 1.8 (Rottbøll & Friis, 2013).

In conclusion, a wide variety of antimicrobials are available on the market to treat respiratory infections. Knowing the bacteria susceptibility to those antimicrobials and the drug pharmacokinetics will help to set the appropriate treatment to achieve a favorable outcome.

3.2. Pleuropneumonia

Actinobacillus pleuropneumoniae (APP) is the causative agent of porcine pleuropneumonia, a worldwide disease with occasional clinical outbreaks that can have a severe economic impact (Gottschalk & Taylor, 2006). Attempts to

control the disease have been made by vaccination, treatment with antibiotics and the establishment of herds free of the infection. Pigs can become asymptomatic carriers of the organism in their tonsils for long periods (Maccinnes & Rosendahl, 1988; Vigre *et al*., 2002), thereby exposing susceptible animals and maintaining the disease in the herd. Moreover, pigs can carry APP in their tonsils for several months without seroconverting (Lavritsen *et al*., 2002). Briefly, the tonsil, or more specifically the palatine tonsil, is the major immunological component of the oropharinx located in the soft palate. This consists of organized lymphoid tissue covered by stratified squamous epithelium but penetrated by branching crypts covered with non-keratinized epithelium. The organized tissue contains B cell follicles and T cells (Pastoret *et al*., 1998). The crypt epithelium is a lymphoepithelium containing goblet cells, microfold cells (M cells) and intraepithelial lymphoid cells (Belz & Heath, 1996). Some bacteria native to the oropharynx may inhabit the tonsils, resulting in subclinical carriers of, for example*, Actinobacillus Pleuroneumoniae* (Macinnes & Rosendahl, 1988; Vigre *et al.*, 2002), *Erysipelothrix rhusiopathiae* (Takahashi *et al*., 1999)*,* salmonellae, or some groups of streptococci (Pastoret *et al*., 1998).

Treatment with antimicrobial drugs should be applied at initial stages of the disease to achieve the best results. A delay in the treatment can imply high mortality or the development of chronic respiratory cases in treated animals. Antimicrobials should be applied parenterally as ill animals may not drink or eat enough to achieve the desired drug concentrations (Pijpers *et al.,* 1990). Depending on each antimicrobial, repeated administration may be done to ensure the appropriate and effective drug levels. The early detection of the symptoms and clinical signs and the immediate therapeutic intervention are keys on the treatment outcome. On the other hand, water medication may be applied to treat the rest of the herd that are still able to drink. Feed medication may be successful if all the animals eat and drink normally. The best results could be obtained with the combination of parenteral and peroral therapy but it must be studied in a case by case situation. In the treatment of respiratory diseases with antimicrobials, clinically recovered animals, does not eliminate the bacteria. Thus, infection can persist on the tonsils or in chronic lung

abscesses of recovered animals and become a source of infection for healthy animals during the rest of the rearing period. It could be a reasonable explanation for the occurrence of relapses at population level.

Some of the antimicrobial agents that showed in vitro activity against APP are: penicillin, amoxicillin, cephalosporins, tetracyclines, streptomycin, gentamicin, erythromycin, thrimethoprim, fluoroquinolones and florfenicol (Gutiérrez-Martín *et al*., 2006). Although β–lactams showed *in vitro* activity (Matter *et al*., 2007), some data suggest a decrease in susceptibility in two of the most used drugs of this family, amoxicillin and ampicillin (Vanni *et al*., 2012). Fluoroquinolone family compounds showed an effective *in vitro* activity against APP (Norcia *et al*., 1999). In the particular case of Italy, it has been described that marbofloxacin has the lowest rate of resistance for this bacteria (Vanni *et al*., 2012). Tulathromycin, a new triamilide antimicrobial of the macrolide family has also been reported as an effective treatment against APP (Hart *et al*., 2006). Florfenicol, a chloramphenicol derivative, has been reported as effective in vitro and with low values of resistance rates in different countries (Priebe & Schwarz, 2003; Gutiérrez-Martín *et al*., 2006; Morioka *et al*., 2008; Vanni *et al*., 2012). Sensitivity tests and antibiogram are highly advisable to avoid failure treatments with antimicrobials.

Attempts to eradicate APP from pig herds have been made with different antibiotics. For example, Fittipaldi *et al.* (2005) used feed medicated with tilmicosin phosphate for 30 days but found that the tonsils of the majority of animals were still PCR-positive 30 days later. Most of the results have been published in case reports describing procedures applied to one or a few farms (Angen *et al.*, 2008). On the other hand, an eradication program that includes sow medication with a fluoroquinolone was successful (Bækbo, 2006). However, any of the previously commented field trials are not supported with the determination of antibiotic concentration in the tonsils (target tissue).

3.3. Glässer disease

Glässer's disease is a swine infectious disease caused by *Haemophilus parasuis* (HP), a Gram-negative bacterium from the Pasteurellaceae family. Glässer's disease is characterized by fibrinous polyserositis, polyarthritis and meningitis in post-weaning pigs, from three to six weeks old. It is frequently developed after stressful events such as weaning, environment changes and pig mixing or as concomitant of other infectious agents (e.g. PRRSv) (Rapp-Gabrielson *et al.,* 2006). HP colonizes the upper respiratory tract of healthy newborn pigs. Different strains with different virulence can be found in the same animal or herd (Harris *et al*., 1969; Cerdà-Cuéllar *et al*., 2010). In this term, the potential virulence of HP can be evaluated by testing the presence of virulence-associated trimeric autotrasporter (*vtaA*) by PCR. Specifically, the presence of group 1 v*taA* genes is the one that is associated with virulence (Olvera *et al*., 2011)

Glässer's disease has an important economic impact in infected herds due to the mortality and the costs of antimicrobial treatment. The disease course is usually short and some of the pigs may die without being treated. Therefore, ill animals should be treated as soon as they shown disease signs using an antimicrobial administered parenterally. Nevertheless, there is no information available in how the antimicrobial treatment affects the colonization and excretion of the bacteria during the weaning period. The antimicrobial of choice to treat parenterally is penicillin although one paper reported a reduction of HP susceptibility to this drug (Rapp-Gabrielson *et al.,* 2006). HP also showed *in vitro* sensitivity to amoxicillin, ampicillin, apramycin, ceftiofur, cephalosporin, clindamycin, doxycycline, enrofloxacin, erythromycin, florfenicol, gentamicin, neomycin, norfloxacin, oxytetracycline, spectinomycin, tetracycline, tiamulin, tilmicosin, trimethoprim/sulphamethoxazole and tylosin. Resistance to antimicrobials shows a huge variability depending on the region. Thus, Spain has bigger rates of antimicrobial resistances in almost all the antimicrobial families when compared to other European countries (Wissing *et al*., 2001; Aaerstrup *et al*., 2004; Nedbalcova *et al*., 2006; Martín de la Fuente *et al*., 2007; Markowska-Daniel *et al*., 2010). Another effective drug of choice would be

peroral amoxicillin, either through the feed or the water. However, best results are obtained if the antimicrobial is administered before clinical signs become apparent.

Preventive measures such as management modifications, control of concomitant diseases, use of antimicrobials and vaccination may be carried out to control the disease. Early segregation to avoid the colonization has been proposed but it was not always successful. Complete elimination of HP was successfully only combining the early segregation (weaning at 7, 14 and 21 days) and high doses of antimicrobials administered parenterally and orally to piglets (Clark *et al.*, 1994). The environment improvement and vaccination has shown some benefits. However, a policy of reducing and minimizing the stressful events and improving the environment in the nursery should be of first importance.

II. HYPOTHESIS AND OBJECTIVES

We are not lost, we're locationally challenged

John M. Ford

Fluoroquinolones, and more specifically MB, are effective against most agents of the porcine respiratory disease complex (PRDC). However, more information is needed about MB and its potential to improve the treatment of these respiratory infections. The main goals of the present thesis was to evaluate and expand the current pharmacological knowledge on the use of MB to treat two of the most common agents of the PRDC which are sensitive to fluoroquinolones, APP and HP, and the way that PK-PD relationships can help with dose optimization. In addition, the potential use of MB to control HP and APP at a population level was explored. Specific objectives of this thesis were:

1. To assess the penetration of marbofloxacin in the pig tonsils in order to evaluate its possible role in the control or eradication of the disease.

2. To define the HP infection dynamics within the herd after the application of a marbofloxacin treatment.

3. To foresee the effect in terms of clinical resolution and the generation of antimicrobial resistance after marbofloxacin treatment at population level taking into account the MIC probability distribution of marbofloxacin against HP and APP and its pharmacokinetic variability.

III. STUDIES

You don’t know much and that’s a fact

Lewis Carroll, Alice’s Adventures in Wonderland

Study I

Marbofloxacin reaches high concentration in tonsil in a dose-dependent fashion.

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Marbofloxacin reaches high concentration in pig tonsils in a dose-dependent fashion

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Marbofloxacin (MB) is a fluoroquinolone widely used in veterinary medicine. This molecule is an organic acid with good tissue penetration. Its volume of distribution exceeds the body water volume, and the degree of binding to plasma proteins is low (Sidhu *et al.*, 2010). It possesses a broad spectrum of activity against mycoplasmata, most Gram-negative and some Gram-positive bacteria as well as some intracellular pathogens such as *Brucella* and *Chlamydia* species, but with limited or no activity against anaerobes (Hannan *et al.*, 1989; Spreng *et al.*, 1995; Appelbaum & Hunter, 2000). This spectrum of activity includes most of the swine respiratory pathogens, including *Actinobacillus pleuropneumoniae* (APP).

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a worldwide disease with occasional clinical outbreaks that can have a severe economic impact (Gottschalk & Taylor, 2006). Attempts to control the disease have been made by vaccination, treatment with antibiotics and the establishment of herds free of the infection. Pigs can become asymptomatic carriers of the organism in their tonsils for long periods (Macinnes & Rosendahl, 1988; Vigre *et al.*, 2002), thereby exposing susceptible animals and maintaining the disease in the herd. Moreover, pigs can carry APP in their tonsils for several months without seroconverting (Lavritsen *et al.*, 2002). Attempts to eradicate APP from pig herds have been made with different antibiotics. For example, Fittipaldi *et al.* (2005) found that the tonsils of the majority of animals were still PCR-positive 30 days later after the use of feed medicated with tilmicosin phosphate. Most of the results have been published in case reports describing procedures applied to one or a few farms (Angen *et al.*, 2008). On the other hand, an eradication programme that includes sow medication with a fluorquinolone was reported to be successful (Bækbo, 2006). However, these field trials are not supported with the determination of antibiotic concentration in the tonsils (target tissue). Thus, the goal of this study was to quantify the MB penetration in tonsils after applying two different MB dose regimens to find out its potential use to eliminate APP from tonsils in carrier animals.

To this end, thirty 2-month-old pigs weighting 17.4–27.1 kg were selected for this study coming from a farm with clinical cases of porcine pleuropneumonia. Animals were clinically healthy when the study began. Pigs received nonmedicated commercial feed ad libitum and had free access to drinking water. Animals were randomly divided into three groups (control, P1 and P2) of ten animals. Each treatment group received Marbocyl® 2% (Vetoquinol laboratory, Lure, France) applied at a dose of 2 mg·MB/kg b.w. for three consecutive days (group P1) and at a dose of 4 mg·MB/kg b.w. every 48 h two times (group P2) intramuscularly. Animals of the control group were sham injected with the same volume of physiological saline. The animals were sacrificed by intravenous administration of pentobarbital sodium twenty four hours after the last administration. Blood sample was taken to obtain serum, and tonsils were collected and frozen at −80 °C until analysis.

The concentration of MB in serum and tonsils was quantified by high performance liquid chromatography (HPLC) according to standard procedures. Briefly, serum samples were extracted with dichloromethane after adding ofloxacin as internal standard. This extraction method is based on a liquid-liquid process. Tonsil tissue was homogenized and treated with a protease. Afterwards, ofloxacin was added as internal standard, and the mixture was also extracted with dichloromethne as previously described for serum samples. HPLC-reversed phase with a C18 stationary phase (analytical column: Merck Lichrospher 100RP18 (250 × 4) mm, 5 μm) with fluorescence detection set to 295 nm for excitation and 500 nm for emission was used. The mobile phase was a mixture of phosphate buffer (pH 2.7),

methanol, acetonitrile, acetic acid and triethylamine (86.5/10/2.2/1/0.3 v/v/v/v/v). The limit of quantification was 0.005 μg/mL for serum and 0.005 μg/gr for tonsil. To prepare standards, control serum and tonsils from animals which had received no treatment were spiked with MB, the spiked standard concentrations ranging from 0.005 to 5 μg/mL or μg/g. Both methods were highly linear with coefficients of correlation of the standard curves (r) better than 0.99. Accuracy and reproducibility were determined from inter-day and intra-day variances of assays with spiked concentrations. For the serum samples, accuracy was within the range of 100–102%, and precision was better than 5%. For tonsil samples, accuracy was within the range of 95–103%, and precision was better than 11%. A nonparametric test (Mann–Whitney) was used to compare the MB concentration achieved in serum and tonsils between the P1 and P2 groups. The SPSS 15.0 software was used (SPSS Inc., Chicago, IL, USA) to carry out this statistical analysis, and the level of significance (α) was set to $P < 0.05$.

Finally, the ratio MB concentration in tonsils was compared with MIC_{90} value (0.03 μg/mL) determined for APP following CLSI (CLSI M31-A2, 2002) recommendations (Valle *et al.*, 2006). The MB tonsil: MIC_{90} ratio calculated did not correspond to the C_{max}/MIC ratio because of that the sample time chosen was 24 h after the intramuscular administration and the Tmax described for pigs after intramuscular administration is 0.8 h (Anonymous, 1997). This ratio of tissue concentrations vs. MIC_{90} values is one of the PK/PD efficacy parameters described for fluoroquinolones (Sarasola *et al.*, 2002).

Average MB serum concentrations were 0.16 and 0.24 μg/mL, 24 h after administering the last Marbocyl® 2% dose for the P1 and P2 group, respectively. Moreover, average MB tonsil concentrations were 0.50 and 0.70 μg/gr for the P1 and P2 group, respectively (Fig. 1). MB concentration was significantly higher for the P2 group than for the P1 group in plasma ($P = 0.01$) and tonsils ($P = 0.009$) Thus, serum and tonsil tissue concentrations increased in a dose-dependent fashion, but the tonsil MB vs. serum MB concentration ratio was close to three independently of the dose administered to the animals. The MB tonsil concentration:APP MIC_{90} ratios were 16.6 and 23.3 for P1 and P2 group, respectively.

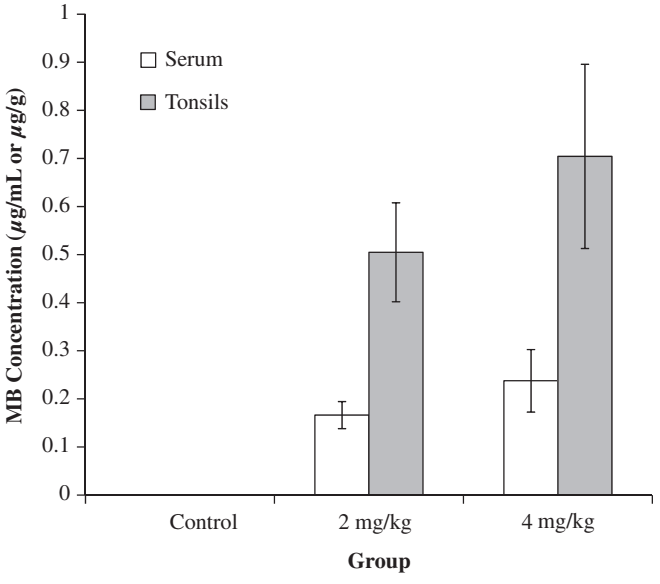

Fig. 1. Mean (± standard deviation) concentration (μg/mL or μg/gr, respectively) of MB in serum (white bars) and tonsil (grey bars) 24 h after the last intramuscular application of marbofloxacin at 2 and 4 mg of MB/Kg administered three times (every 24 h) and two times (every 48 h), respectively, in 10 pigs for experimental group.

The main goal of this study was to quantify the penetration of MB in pig tonsils. It would have been ideal to define its pharmacokinetic tonsil profile using, at least, samples from five different times as it has been described for moxifloxacin in humans (Esposito *et al.*, 2006). However, the quantification of this antibiotic in tonsil require the use of the complete tonsil and, consequently, to sacrifice the animals. Thus, it was decided to use a representative number of animals (10) in one single sample time to minimize the number of animals used for welfare reasons. The sample time (24 h after intramuscular administration) was chosen to allow a distribution equilibrium even whether the tonsil would behave as a deep tissue for antibiotic penetration in pigs.

Presented data show that MB achieves a good penetration into tonsillar tissue, which is comparable with tonsil/plasma ratios reported for other fluoroquinolones such as the ratio of 1.5–1.9 for ciprofloxacin, from 1 to 8 h after oral or intravenous doses of 200–500 mg; 2.02–2.08 for levofloxacin, from 1 to 9 h after single oral doses of 100 or 200 mg; and 1.4 for ofloxacin, 2 h after a single oral dose of 200 mg (Falser *et al.*,1988; Fish & Chow, 1997). Tonsil/plasma ratio observed for MB was also very similar to that of moxifloxacin in humans as described by Esposito *et al.* (2006). MB tissue/plasma ratio, for other pig tissues at steady-state (4 h after a intramuscular dose of 2 mg of MB/body weight), such as the lung (1.8), liver (1.9), kidney (3.8), muscle (1.7) and skin (1), is equal or lower than the value observed for pig tonsils (Anonymous, 1997) clearly showing that tonsil did show a similar distribution pattern compared to most studied pig tissues.

In PK/PD relationships for fluoroquinolones, the C_{max}:MIC ratio has been shown to have particular utility in determining optimal activity against Gram-negative micro-organisms (Drusano *et al.*, 1993; Sarasola *et al.*, 2002). A C_{max}:MIC ratio of >8 and an AUC_{0-24}:MIC ratio of >100 have been recommended to prevent resistance selection (Dudley, 1991; Thomas *et al.*, 2001). The MB tonsil:MIC ratio described is above the threshold value (10) that is associated with clinical efficacy for all the doses studied (Drusano *et al.*, 1993; Sidhu *et al.*, 2010). Obviously, the information provided here should be confirmed by trials that would study the eradication of APP form tonsils of infected animals.

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Study II

Effect of marbofloxacin on *Haemophilus parasuis* nasal carriage.

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Effect of marbofloxacin on *Haemophilus parasuis* nasal carriage

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ABSTRACT

Haemophilus parasuis is a colonizer of the upper respiratory tract and the causative agent of Glässer's disease in swine. This study focused on the nasal carriage of *H. parasuis* after treatment with marbofloxacin. Three marbofloxacin treatments (three doses of 2 mg/kg body weight [bw] every 24 h, two doses of 4 mg/kg bw every 48 h and 8 mg/kg bw in one single shot) were used and all of them reduce significantly ($p < 0.05$) the nasal carriage of *H. parasuis* as compared to control animals. Moreover, *H. parasuis* was not detected in the nasal cavities of piglets after administering the highest dose. The effect of a dose of 8 mg marbofloxacin/kg bw in one shot was further studied in a farm with clinical cases of Glässer's disease using a longitudinal study. Statistically significant reduction of nasal carriage of *H. parasuis* was detected during the first week after treatment in comparison with the control group. However, a clear relationship between the minimum inhibitory concentration (MIC) of the different strains, their putative virulence or the treatment group (antibiotic or control) from which they were isolated was not detected. Finally, the effect induced by the antibiotic treatment on the bacterial strains seemed to be transitory, since diverse *H. parasuis* strains (with high and low marbofloxacin MICs) were observed 7 days after finishing the treatment.

1. Introduction

Haemophilus parasuis is a Gram-negative bacterium, member of the family Pasteurellaceae, which produces fibrinous polyserositis, polyarthritis and meningitis (Glässer's disease) in pigs (Rapp-Gabrielson et al., 2006). This bacterium is found in the upper respiratory tract of healthy pigs, which are colonized very early after birth by different genotypes of variable virulence (Harris et al., 1969; Cerdà-Cuéllar et al., 2010). Glässer's disease has an important economic impact in affected herds due to the losses caused by the mortality and/or the cost of the antimicrobial treatments necessary to control the disease.

Marbofloxacin is a second-generation fluoroquinolone only used in veterinary medicine. It possesses a broad spectrum activity against *Mycoplasma*, most Gram negative and some Gram-positive bacteria (Appelbaum and Hunter, 2000). This spectrum of activity includes most of the swine respiratory pathogens, including *H. parasuis* (Vallé et al., 2006). Fluoroquinolones conform to concentration dependency against Gram-negative bacteria and achieve values for specific pharmacokinetics and pharmacodyamics parameters are recommended to prevent bacterial growth during treatment and resistance selection (Dudley, 1991; Thomas et al., 2001).

To our knowledge, there is no information available on how antibiotic treatment affects the nasal carriage of *H. parasuis*. Taking into account that fluoroquinolones are usually used to treat respiratory diseases in pigs, the goals

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of the present work are to study the nasal carriage of *H. parasuis* in pigs treated with different posology regimes of marbofloxacin and how these treatments may affect the presence of different strains of this microorganism before and after the treatment under field conditions.

2. Materials and methods

Two different studies were carried out to meet these goals. They are identified as A and B. Study A evaluated the effect of three different marbofloxacin posology regimes (three doses of 2 mg/kg body weight [bw] every 24 h, two doses of 4 mg/kg bw every 48 h and 8 mg/kg bw in one single shot) in nasal carriage of piglets by *H. parasuis*. The effect of the antimicrobial treatment was determined 24 h after the last marbofloxacin administration. In study B, the nasal carriage of *H. parasuis* in piglets treated with marbofloxacin at 8 mg/kg bw in one single shot was evaluated at different time points.

2.1. Study A: effect of 3 different marbofloxacin posology regimes on H. parasuis carriage

The study was carried out in a commercial farm without clinical cases of Glässer disease. Forty 2-month-old pigs weighting 17.4–27.1 kg were used in this study. Animals were clinically healthy when the study began. Pigs received non-medicated commercial feed ad libitum and had free access to drinking water. Animals were housed in a conventional farm under field conditions in pens containing 13 piglets by pen. The space available for the animals was 0.75 m^2/pig. This density was considered adequate under commercial conditions. The building was equipped with manual mechanisms to control ventilation. Animals were ear tagged with unique numbers and were randomly divided into four groups (control, P1, P2 and P3). Group P1 received Marbocyl 2% (Vetoquinol Laboratory, Lure, France) applied intramuscularly at a dose of 2 mg marbofloxacin/kg bw every 24 h for three consecutive days. Group P2 received Marbocyl 2% intramuscularly at a dose of 4 mg marbofloxacin/kg bw twice with a 48 h interval between treatments. Group P3 received Marbocyl 10% (Vetoquinol Laboratory, Lure, France) at a dose of 8 mg marbofloxacin/kg bw in a single shot intramuscularly. In this later group, Marbocyl 10% was used to reduce the volume injected and the possible adverse reactions such as pain and edema at the injection site. These doses were selected taking into account the summary of product characteristics of Marbocyl® and the most frequently used extra-label posology regimens in use under field conditions (JM Caballero, Laboratorios Vétoquinol, Spain, Personal communication). Animals of the control group were sham injected intramuscularly with the same volume of physiological saline. All the animals housed in the same pen received the same treatment (control, P1, P2 or P3) but only 10 out of 13 were sampled to carry out microbiological determinations. This experimental design was chosen to mimic as much as possible the normal situation under field conditions.

Nasal swabs were taken 24 h after the last antibiotic administration, and transported under refrigeration to the laboratory.

No concurrent medications were administered to the animals during the course of the study.

2.2. Study B: nasal colonization of H. parasuis in piglets treated with marbofloxacin at 8 mg/kg bw in one single shot

The study was carried out in a farm with clinical cases of Glässer's disease, with 1500 sows in a farrow-to-finish production system. A total of 300 4-week-old crossbred pigs were used in this study (150 animals per group). Animals were clinically healthy when the study began. Animals were housed in pens containing 25 animals per pen. The space available per animal was 0.36 m^2/animal. This density was considered adequate under commercial conditions. The building was equipped with automatic mechanism to control ventilation and temperature. Pigs were fed and had water available ad libitum. The feed was distributed in hoppers (one per pen) and the water was supplied through an automated system. All the pigs included in the study received non-medicated feed, which was normally applied under commercial conditions in this farm. Feed was stored at room temperature. Animals were randomly divided in two groups, which were placed in independent rooms with independent ventilation. Treatment group received Marbocyl® 10% (Vétoquinol Laboratory, Lure, France) applied intramuscularly at a dose of 8 mg/kg bw in one shot. Animals of the control group were sham injected intramuscularly with the same volume of physiological saline. Treatments were applied at the beginning of the nursery period. Piglets included in the trial were clinically monitored and mortality was also recorded. A subpopulation of 20 piglets of each group were randomly selected and tagged. Nasal swabs from those selected piglets were taken on days 0, 1, 7, 14 and 28 and transported under refrigeration to the laboratory.

Piglets were observed daily for general health conditions. No concurrent medications were administered to the animals during the course of the study.

2.3. H. parasuis isolation and identification

Collected swabs were plated on chocolate agar (bioMérieux, Madrid, Spain). After 2–3 days at 37 °C with 5% CO_2, all *H. parasuis*-like colonies were selected and subcultured for identification and further analysis. The swabs were also processed for DNA extraction with the Nucleospin blood kit (Macherey-Nagel) following manufacturer instructions and the extracted DNA was used in a species-specific PCR to identify *H. parasuis* (Oliveira et al., 2001).

2.4. Characterization of H. parasuis isolates

Two different PCR were used for characterization of *H. parasuis* isolates: enterobacterial repetitive intergenic consensus (ERIC)-PCR for determination of the different strains in the animals (Olvera et al., 2006) and the virulence-associated trimeric autotransporter (*vtaA*) PCR for determination of the putative virulence of the strains (Olvera et al., 2011).

Purified DNA from each *H. parasuis* isolate was quantified by spectrometry and 100 ng were used as template in ERIC-PCR. The technique followed a previously published protocol (Olvera et al., 2006). Bands from 4000 to 100 bp were used for comparison of the different isolates.

Selected isolates were tested with the *vtaA*-PCR as previously described (Olvera et al., 2011). The group 1 *vtaA* is associated with the virulence of the strains.

2.5. MIC determination

Antimicrobial susceptibility tests were performed using the agar dilution method according to CLSI guideline M31-A3 Clinical and Laboratory Standards Institute (2011) with some modifications. Briefly, a MacFarland suspension of 1 was prepared from a 24 h culture on chocolate agar from each isolate. Each bacterial suspension was spread on chocolate agar plates loaded with 2-fold dilutions (from 16 to 0.0078 μg/mL) of marbofloxacin. Plates were subsequently incubated for 24–48 h at 37 °C with 5% CO_2. A plate without marbofloxacin was included as growth control for each isolate. In both studies, the MIC was determined for isolates from day 0 and, in study B, the MIC was also determined 24 h after finishing the antimicrobial treatment.

2.6. Pharmacokinetics/pharmacodyamics (PK/PD) parameters

A great deal of information is now available on the pharmacokinetics (PK) and pharmacodynamics (PD) relationships for fluoroquinolones. The main pharmacokinetic parameters to take into account for fluoroquinolones are the maximum drug concentration observed in serum (C_{max}) after administering a dose and the area under the curve (AUC) that is a direct measure of the exposure of the organism to a drug after its administration. This latter parameter is calculated as the product of the plasma concentration and the time during the first 24 h after its administration. Finally, the key pharmacodynamic parameter for antimicrobials is the MIC.

Pharmacokinetic/pharmacodynamic (PK/PD) interactions for antimicrobial drugs result in gross observable readouts in administered animals including clinical improvement, growth promotion and adverse reactions. In the particular case of fluoroquinolones,, they conform to concentration dependency against Gram negative bacteria and the ratio between the maximum drug concentration observed in serum (C_{max}) versus the MIC (C_{max}/MIC) and the ratio between the AUC after a single dose within 24 h and MIC (AUC_{0-24}/MIC), have been shown to have particular utility in determining their optimal activity (Drusano et al., 1993; McKellar et al., 2004). Finally, a C_{max}/MIC and AUC_{0-24}/MIC ratio higher than 8 and 100, respectively, have been shown to prevent bacterial growth during treatment and to prevent resistance selection (Dudley, 1991; Thomas et al., 2001).

In our case, C_{max}/MIC and AUC_{0-24}/MIC were calculated (McKellar et al., 2004) using the highest MIC determined in each study and the marbofloxacin pharmacokinetic data available in the public domain. Briefly, a C_{max} and AUC_{0-24} values of 1.4 μg/mL and 12 μg h/mL, respectively, were used for a dose of 2 mg of marbofloxacin/kg bw administered by intramuscular route. Pharmacokinetic values for higher doses (4 and 8 marbofloxacin/kg bw) have been extrapolated taking into account the linear pharmacokinetic behavior of this molecule in pigs (Marbocyl®. Marbofloxacin Reference Book, Vétoquinol). Thus the extrapolated values for C_{max} and AUC_{0-24} were 2.8 μg/mL and 24 μg h/mL for the dose of 4 mg marbofloxacin/kg bw and 5.6 μg/mL and 48 μg h/mL for the 8 mg marbofloxacin/kg bw, respectively.

2.7. Statistical analysis

All statistical analyses were carried out using the SAS system V.9.1.3 (SAS institute Inc., Cary, NC, USA). For all analyses, pig was used as the experimental unit. The significance level (α) was set at 0.05. Differences in the percentage of *H. parasuis* positive animals between groups were compared using a chi-square test.

3. Results

3.1. Study A: effect of 3 different MB posology regimes on the H. parasuis colonization

Animals did not show any clinical symptom during the trial and any adverse reactions after the marbofloxacin treatment. Significant differences ($p < 0.05$) in the percentage of *H. Parasuis* PCR positive animals in nasal cavity between control group and groups that received different marbofloxacin posology regimes were observed 24 h after finishing the antibiotic treatment (Fig. 1). The three marbofloxacin treatments reduced significantly ($p < 0.05$) the nasal colonization by *H. parasuis* as compared to control animals (close to 90% of positive animals). Groups P1 and P2 showed the same low level of *H. parasuis* detection in the pig nostrils after the treatment (10% of positive animals)

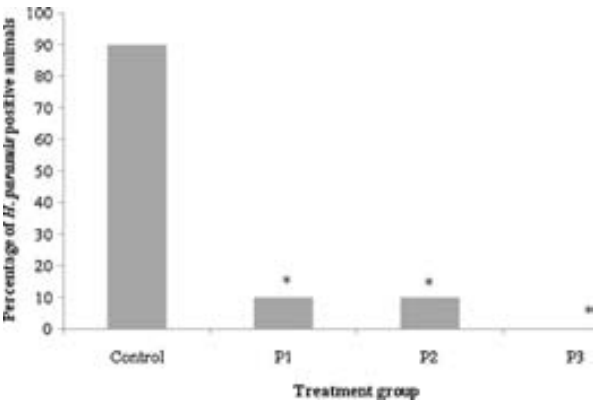

Fig. 1. Percentage of *H. parasuis* positive animals by specific PCR 24 h after finishing the marbofloxacin treatment. Control group received no antimicrobial. P1 was treated with three doses of 2 mg marbofloxacin/kg bw administered intramuscularly every 24 h; P2, with two doses of 4 mg marbofloxacin/kg bw administered intramuscularly every 48 h; and P3, with 8 mg marbofloxacin/kg bw in a single shot. Asterisk indicates significant difference ($p < 0.05$) versus control.

Table 1
H. parasuis detection by PCR and bacterial culture in each animal from study B.

Animal #	Day 0 PCR/culture	Day 1 PCR/culture	Day 7 PCR/culture	Day 14 PCR/culture	Day 28 PCR/culture
Control group					
41	+/+	+/+	+/−	+/−	+/−
42	+/−	−/−	−/−	−/−	+/+
43	+/−	−/−	+/+	+/+	+/+
44	+/−	+/−	+/+	+/−	+/+
45	+/−	−/−	+/−	+/−	+/−
46	+/−	+/−	+/−	+/−	ND
47	+/−	+/−	ND	ND	ND
48	+/−	+/−	ND	ND	ND
49	+/−	+/−	+/−	+/−	+/−
50	+/−	+/−	+/+	+/−	+/−
51	+/−	−/−	+/−	+/−	+/−
52	+/−	+/−	−/−	−/−	+/−
53	+/+	+/+	+/−	+/−	+/−
54	+/−	+/−	−/−	−/−	+/−
55	+/+	+/−	−/−	−/−	+/−
56	+/−	−/−	+/−	+/−	+/−
57	+/+	+/−	+/+	+/−	+/−
58	+/−	+/−	+/+	+/−	−/−
59	+/+	+/−	+/−	−/−	+/+
60	−/−	−/−	+/−	−/−	+/−
Treatment group					
81	+/−	+/−	−/−	−/−	+/−
82	+/+	−/−	−/−	+/−	+/−
83	+/−	−/−	−/−	−/−	+/−
84	+/−	+/−	+/+	+/−	+/+
85	+/+	+/+	−/−	−/−	+/−
86	+/+	+/−	+/−	+/−	+/−
87	+/−	−/−	−/−	+/−	+/−
88	+/−	−/−	−/−	+/−	+/−
89	+/−	−/−	+/−	+/−	+/−
90	+/−	−/−	−/−	+/−	+/−
91	+/+	−/−	+/+	+/−	+/−
92	+/−	−/−	+/−	+/−	+/−
93	+/−	−/−	−/−	−/−	+/−
94	−/−	−/−	−/−	−/−	+/+
95	+/+	−/−	+/−	+/−	+/−
96	+/−	−/−	+/+	+/−	+/+
97	+/+	−/−	−/−	−/−	+/+
98	+/+	−/−	−/−	−/−	+/+
99	+/−	−/−	−/−	−/−	+/+
100	+/+	−/−	−/−	−/−	+/−

ND: no determined, due to the death of the animals.

whereas *H. parasuis* was not detected in the nasal cavities of piglets from the P3 group (single shot of 8 mg marbofloxacin/kg bw). Finally, the marbofloxacin MIC of the different isolates recovered in this study ranged from 0.0156 to 0.25 μg/mL.

3.2. Study B: *H. parasuis* colonization of piglets treated with marbofloxacin 8 mg/kg bw in one single shot

The effect of marbofloxacin on long-term nasal carriage of *H. parasuis* was also studied. Nasal swabs from piglets treated with a single shot of 8 mg/kg marbofloxacin were examined by PCR to detect *H. parasuis*.

It was not observed clinical signs compatible with glässer disease throughout the trial and mortality was not significantly different between the control (7 out of 150 piglets) and the group that receive the antibiotic treatment (6 out of 150 piglets). Moreover, from the subpopulation that was selected for sampling (20 animals in both groups), three animals in the control group died during the experiment; 2 animals died the first day of the trial and 1 animal died 14 days after the beginning of the trial. The three animals were housed in the same pen and showed nervous symptoms. Unfortunately, the cause of death of these animals could not be determined and they were excluded from the study. Therefore, 17 and 20 animals were used for sampling from the control and treatment group throughout the trial, respectively.

The results for the PCR and bacterial isolation for each animal are shown in Table 1. As expected, the PCR method was more sensitive than the *H. parasuis* isolation from the nasal cavities. The percentage of positive animals in the *H. parasuis* PCR at days 0, 1, 7, 14 and 28 is shown in Fig. 2. Significant differences between both groups were found at 1 and 7 days after treatment. However, at days 14 and 28 post-treatment the difference in *H. parasuis* colonization between the groups disappeared.

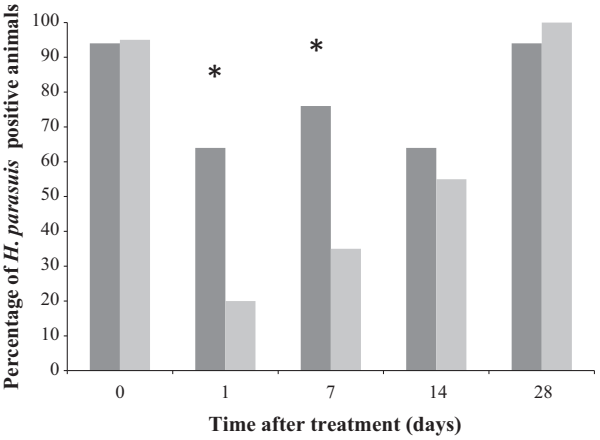

Fig. 2. PCR detection of *H. parasuis* in nasal swabs from piglets treated with 8 mg marbofloxacin/kg bw in one single shot. Nasal swabs were taken at days 0, 1, 7, 14 and 28 after treatment (light gray bars). Non-treated animals were also tested and are included as control (dark gray bars). Asterisk indicates significant differences ($p < 0.05$) between treatment and control group.

Table 2
H. parasuis strains isolated throughout the trial and their main characteristics.

Strain	Isolation group[a]	Isolation time[b]	MIC (mg/L)	*vtaA*-1 PCR[c]
A	T	7	ND	–
B	T	7	ND	–
C	T/C	0, 1	2	+
D	T/C	0, 7	1	+
E	T	28	ND	+
F	T/C	0, 7	0.25	–
G	T	0	0.5	–
H	C	0	0.5	–
I	C	7	ND	–
J	T/C	7	ND	–
K	C	14, 28	ND	–
L	T	7	ND	+
M	T/C	0, 7, 28	1	–
N	T	28	ND	–
O	T	28	ND	+
P	T	28	ND	+
Q	T	0, 7	2	+

[a] C, control; T, treated with marbofloxacin or T/C, both.
[b] Time after treatment in days.
[c] *vtaA*-1 positive PCR is indicative of virulence of *H. parasuis* strains.

All the *H. parasuis* isolates from this study (62 isolates) were analyzed by ERIC-PCR to determine the different strains present in animals. With this technique, 17 different strains were determined within the 62 isolates. A code letter was assigned to each strain (letters from A to Q) and their main characteristics are detailed in Table 2, including the MIC for marbofloxacin for the strains isolated on days 0 and 1 of treatment (7 strains in total). Thus the MICs calculated ranged from 0.25 to 2 μg/mL. A clear relationship between the MIC of the different strains, their putative virulence and the treatment group from which they were isolated was not detected.

PK/PD parameters obtained in study A and B are shown in Table 3. The parameters were calculated using the highest MIC found in each study (0.25 μg/mL in study A and 2 μg/mL in study B). PK/PD parameters calculated were above the threshold value for clinical efficacy of fluoroquinolones (C_{max}/MIC > 8 and AUC_{0-24}/MIC > 100) when a dose of 4 mg/kg bw every 48 h (C_{max}/MIC = 11.2 and AUC_{0-24}/MIC = 96) and 8 mg/kg bw in one single shot (C_{max}/MIC = 22.4 and AUC_{0-24}/MIC = 192) were administered in study A. However, these parameters were below the threshold value for clinical efficacy in study B even

Table 3
Marbofloxacin C_{max}, AUC_{0-24} and its PK/PD parameters (C_{max}/MIC and AUC_{0-24}/MIC) were calculated using the highest MIC found in each study (0.25 μg/mL in study A and 2 μg/mL in study B) and marbofloxacin pharmacokinetic data available in the public domain (see Section 2 for details). PK/PD parameters above the threshold values associated with clinical efficacy for fluoroquinolones (C_{max}/MIC > 8 and AUC_{0-24}/MIC > 100) are highlighted.

Marbofloxacin dose	2 mg/kg	4 mg/kg	8 mg/kg
C_{max} (μg/mL)	1.4	2.8	5.6
AUC_{0-24} (μg h/mL)	12	24	48
$C_{max}/MIC_{Study\ A}$	5.6	11.2	22.4
$AUC_{0-24}/MIC_{Study\ A}$	48	96+	192
$C_{max}/MIC_{Study\ B}$	–	–	2.8
$AUC_{0-24}/MIC_{Study\ B}$	–		24

+, very close to the threshold value.

though only the highest dose was used in this case. Thus, C_{max}/MIC and AUC_{0-24}/MIC were 2.8 and 24, respectively.

4. Discussion

A marbofloxacin dose-effect was observed in the *H. parasuis* nasal carriage. In addition, a time-effect was also detected at the dose of 8 mg/kg bw marbofloxacin. Thus, this treatment produced a reduction of the presence of *H. parasuis* during the first week after treatment. This result indicates that this treatment may be useful to control the disease produced by *H. parasuis*. Nevertheless, further transmission studies would be necessary to address this issue.

The PK/PD values were calculated assuming that marbofloxacin concentration in the pig nasal mucosa is, at least, very similar to the serum concentration observed at the same kinetic time. This assumption is based on: (1) good bioavailability and wide tissue distribution reported on fluoroquinolones (Martínez et al., 2006), (2) pharmacokinetic data of marbofloxacin in pigs (Marbocyl®. Marbofloxacin Reference Book, Vétoquinol) and (3) nasal and bronchial concentration of marbofloxacin, danofloxacin and enrofloxacin described in calves (McKellar et al., 1999; Banting et al., 1997). Only the dose with the expected effective PK/PD value, above the threshold values associated with clinical efficacy for fluoroquinolones (C_{max}/MIC > 8 and AUC_{0-24}/MIC > 100), was effective in reducing the quantity to bacteria to an undetectable level by PCR in study A. In the case of study B, this PK/PD values were not reached due to the high marbofloxacin MIC present in this farm. Differences in MIC values between both farms can be explained by the use of enrofloxacin to treat Glässer's disease in former respiratory outbreaks in farm B. It is well-known that fluoroquinolones can lead to cross-resistance among them (Garau, 2000) and it is not uncommon to find a medium-high level of *H. parasuis* resistances against fluoroquinolones (De la Fuente et al., 2007; Xu et al., 2011) since they are widely used to treat respiratory diseases. In summary, in both studies, PK/PD parameters are a useful tool to foresee clinical efficacy of marbofloxacin treatment taking into account detection of *H. parasuis* in nasal cavity as end-point.

The antibiotic treatment is modifying, in some way, the strains of *H. parasuis* toward a subpopulation with the highest antimicrobial MIC immediately after applying the antibiotic treatment, as it has been described by other authors (Drlica and Zhao, 2007; Roberts et al., 2008) but, in this particular case, this effect seemed to be transitory because a diverse *H. parasuis* strain population was observed 7 days after finishing the treatment. Moreover, 7 out of the 17 strains may be considered virulent, since they were found positive in the *vta*A-1 PCR. Nevertheless, a clear relationship between the MIC of the different isolated strains and their putative virulence was not observed. This result clearly suggests that antimicrobial treatment is not necessarily a driving force for the selection of virulent strains, in agreement with similar findings in *H. influenzae* (Samuelson et al., 1995).

In conclusion, the amount of *H. parasuis* can be reduced in the nasal cavity during short periods of time after the application of marbofloxacin intramuscularly although a complete elimination of the bacteria was not possible.

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Study III

Pharmacokinetic/pharmacodynamic evaluation of marbofloxacin in the treatment of *Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* infections in nursery and fattener pigs using Monte Carlo simulations.

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Pharmacokinetic/pharmacodynamic evaluation of marbofloxacin in the treatment of *Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* infections in nursery and fattener pigs using Monte Carlo simulations

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Vilalta, C., Giboin, H., Schneider, M., El Garch, F., Fraile, L. Pharmacokinetic/pharmacodynamic evaluation of marbofloxacin in the treatment of *Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* infections in nursery and fattener pigs using Monte Carlo simulations. *J. vet. Pharmacol. Therap.* doi: 10.1111/jvp.12134.

This study evaluated the theoretical clinical outcome of three marbofloxacin posology regimens in two groups of pigs (weaners and fatteners) for the treatment of *Actinobacillus pleuropneumoniae* (App) and *Haemophilus parasuis* (Hp) infection and the appearance of resistant bacteria due to the antibiotic treatment. The probability of target attainment (PTA) for pharmacokinetic/pharmacodynamics (PK/PD) ratios associated with clinical efficacy and with the appearance of antimicrobial resistance for fluoroquinolones at each minimum inhibitory concentration (MIC) or mutant prevention concentration (MPC) were calculated, respectively. The cumulative fraction of response (CFR) was calculated for the three posology regimens against App and they ranged from 91.12% to 96.37% in weaners and from 93% to 97.43% in fatteners, respectively. In the case of Hp, they ranged from 80.52% to 85.14% in weaners and from 82.01% to 88.49% in fatteners, respectively. Regarding the PTA of the PK/PD threshold associated with the appearance of antimicrobial resistance, results showed that marbofloxacin would prevent resistances in most of the animals up to the MPC value of 1 μg/mL.

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INTRODUCTION

Marbofloxacin is a third generation fluoroquinolone widely used in veterinary medicine. Its properties of rapid absorption, good distribution and broad spectrum against most of the swine respiratory pathogens, such *as Haemophilus parasuis* (Hp) and *Actinobacillus pleuropneumoniae* (App), make it a good candidate to deal with a respiratory outbreak due to any of these pathogens.

The major issues of practitioners when treating a large population of animals are to maximize the likelihood of a favourable clinical outcome at population level and to minimize the appearance and development of antimicrobial resistance that could affect future treatments. Pharmacokinetic (PK) and pharmacodynamic (PD) models are a useful tool to foresee clinical efficacy and it could be also used to design and choose the right antimicrobial therapy (Mckellar *et al.*, 2004).

Currently, a great amount of information is available on the pharmacokinetics and pharmacodynamics of fluoroquinolones and the relationship between PK and PD parameters that could be associated with the clinical outcome. The ratios between the area under the curve during the first 24 h and the minimum inhibitory concentration ($AUC_{0\text{-}24}$/MIC) and between the maximum concentration and MIC (C_{max}/MIC) correlate well with successful therapeutic resolution when fluoroquinolones are used to cope with an infection. Thus, a threshold of $AUC_{0\text{-}24}$/MIC of >125 h and C_{max}/MIC of >10 would correlate with successful therapeutic outcome according to literature (Toutain *et al.*, 2002; Mckellar *et al.*, 2004) for fluoroquinolones. Nevertheless, these ratios would not be the most appropriate for the prediction of bacterial resistance. In this case, a better marker to describe the likelihood of appearance of antimicrobial resistance is the ratio between the $AUC_{0\text{-}24}$ and the mutant prevention concentration (MPC) (Zhao & Drlica, 2008). Regarding $AUC_{0\text{-}24}$/MPC, the study of Cui *et al.* (2006) established that a value of $AUC_{0\text{-}24}$/MPC above 25 h restricts the acquisition of resistances in a *Staphylococcus aureus* infection (a gram-positive bacterium). Similar values were found by Olofsson *et al.* (2006), in an *in vitro* study, and Ni *et al.* (2013), in an *in vivo* study (rabbit model), where a ratio $AUC_{0\text{-}24}$/MPC>22 h and >20 h were

established to prevent resistance appearance in the case of a *Escherichia coli* infection (a gram-negative bacteria), respectively.

The use of Monte Carlo simulation (MCS) takes into account the variability of the drug PK and the probability distribution of the bacterial MIC to make predictions of the likely result of different therapeutic approaches, using different antimicrobial dosage regimens. To achieve this goal, it is taken into account the threshold values for PK/PD parameters that correlate with clinical efficacy (Roberts *et al.*, 2011). Thus, MCS could be a useful tool to assess and foresee the probability of a favourable outcome of an antibiotic treatment in a large population of animals. This same approach could be used to foresee the appearance of the antimicrobial resistance taking into account the threshold values for PK/PD parameters associated with this event.

The main objective of this work was to evaluate the usefulness of three marbofloxacin posology regimens against Hp and App taking into account their PK and PD variability. Thus, it was assessed the probability of achieving the threshold PK/PD parameters associated with clinical efficacy and with the appearance of antibiotic resistance for marbofloxacin in two pig groups (weaners and fatteners) usually treated with this antibiotic.

MATERIALS AND METHODS

Marbofloxacin PK data and dose selection

Marbofloxacin PK data for weaner and fattener pigs have been recently published by Schneider *et al.* (2014) and this information have been used with permission of the authors for this research work. Briefly, marbofloxacin pharmacokinetic parameters were determined using compartmental analysis with the WinNonlin software version 5.0.1 (Pharsight Corporation, St Louis, MO. USA) in 10 weaners and seven fattener pigs. Pharmacokinetic profile fitted better to a bicompartmental and monocompartmental model for weaners and fatteners, respectively. One animal was excluded from the weaners due to abnormal values for the simulations.

To carry out the pharmacokinetic and pharmacodynamic evaluation, the doses of 2, 4 and 8 mg/kg for marbofloxacin were selected according to the most frequent posology regimens in use under field conditions. As previously commented, the pharmacokinetic data came from a previous study with a dose of 8 mg/kg bw and the data for the 2 and 4 mg/kg bw doses were inferred taking into account the marbofloxacin dose proportionality as described by Schneider *et al.* (2014). It must be highlighted that these doses are usually applied in different posology regimens in daily practice (2 mg/kg bw three times each 24 h, 4 mg/kg bw twice each 48 h and 8 mg/kg bw in one single shot).

Microbiological data

MIC distribution. MIC distribution was extracted from a poster communication presented at the 4th European Symposium of Porcine Health Management (ESPHM) in 2012 (Giboin *et al.*, 2012). MIC was determined as explained elsewhere (Meunier *et al.*, 2004; Kroemer *et al.*, 2012).

MPC determination. The mutant prevention concentration (MPC) corresponds to the first antibiotic concentration at which no bacterium was recovered when 10^{10} cells were applied to agar plates containing 2-fold increasing antibiotic concentration (Blondeau *et al.*, 2001). Simply, a MPC is an MIC determination with a large inoculum (Mouton *et al.*, 2005). The MPC was determined as described by Blondeau *et al.* (2001) with slight modifications. Briefly, each strain was grown overnight (20 to 24 h) on ten plates of Mueller Hinton (MH) agar supplemented with 5% lysed horse blood and 20 μg/mL β-Nicotinamide adenine dinucleotide at 35 ± 2 °C with 5 ± 2% CO_2. Two mL of MH broth was then added to each plate, spread and pooled to give 20 mL of bacterial suspension. After a centrifugation for 30 min at 5000 ***g***, the supernatant was removed and the remaining pellet was resuspended in 3 mL of MH broth. 0.2 mL of the bacterial suspension (around 10^{10} cells) were spread onto supplemented MH agar plates containing appropriate marbofloxacin concentrations (0.002–8 μg/mL). Plates were incubated at 35 ± 2 °C in air supplemented with 5 ± 2% CO_2 and growth observed at 24 and 48 h. MPC was recorded as the lowest antibiotic concentration that allowed no growth. Due to the complexity of this determination, it was only feasible to carry out this determination in six App and two Hp strains.

PK/PD analysis and Monte Carlo simulation (MCS)

The MCS were performed with Oracle Crystal Ball V.11.1.2.0.00. (Oracle Corporation, Redwood Shores, CA, USA). Two sets of simulations were performed, one for the weaners, using the following formula for the bicompartimental model after intramuscular administration:

$$C(t) = \frac{F \times D \times K01}{V} \times \left(\frac{K21 - \alpha}{(K01 - \alpha) \times (\beta - \alpha)} \times e^{-\alpha t} + \frac{K21 - \beta}{(K01 - \beta) \times (\alpha - \beta)} \times e^{-\beta t} - \frac{K21 - K01}{(\alpha - K01) \times (K01 - \beta)} \times e^{-K01 t} \right)$$

where F is the bioavailability, D is the dose of antibiotic administered, K01 is the absorption rate constant, V is the distribution volume, K21 is an intercompartmental micro-rate constant, α and β are elimination macro-rate constants and t is a given time.

In the case of the fatteners, it was used a monocompartimental model after intramuscular administration:

$$C(t) = \frac{F \times D \times K01}{V \times (K01 - K10)} \times (e^{-K10t} - e^{-K01t})$$

where F is the bioavailability, D is the dose of antibiotic administered, K01 is the absorption rate constant, V is the distribution volume, K10 is the elimination rate constant and t is a given time.

Each simulation set was performed with 10000 simulated PK profiles. The pharmacokinetic values and adjustments used in the models are shown in Table 1 for the bicompartmental model in the case of weaners and in Table 2 for the monocompartmental model in the case of fatteners. The weight was estimated in 25 kg for piglets and 55 kg for fatteners. For the calculations, marbofloxacin concentrations were simulated over 24 h with a step of 0.1 h. $AUC_{0\text{-}24}$ was calculated using the linear trapezoidal mode for each one of the simulated PK profiles.

The following parameters were calculated to foresee the clinical outcome:

a) Probability of target attainment (PTA) (Mouton *et al.*, 2005) in the simulated population taking into account the PK/PD threshold values of $AUC_{0\text{-}24}$/MIC>125 h and C_{max}/MIC>10 for each MIC point calculated ranging in geometric progression from 0.002 to 8 μg/mL.

b) The cumulative fraction of response (CFR) (Mouton *et al.*, 2005). It is the expected population probability to reach the threshold values of $AUC_{0\text{-}24}$/MIC>125 h or C_{max}/MIC>10 taking into account the probability of the MIC strain distribution. A CFR≥90% was considered optimal against a bacterial population, whereas a CFR ≥80% but ≤90% was associated with moderate probabilities of success (Bradley *et al.*, 2003). This is the most practical parameter for practitioners.

Table 1. Pharmacokinetic parameters used in the bicompartmental model for weaners

Parameter	Distribution	Distribution parameters (coefficient of variation%)
Bioavailability (F)	Beta PERT	Min: 0.87; most likely: 0.93; max:0.99
Distribution Volume (Vd)	Log normal	X:1.58; SD: 0.23 (14.6)
K01	Log normal	X:5.85; SD:0.95 (16.23)
K21	Log normal	X:0.18; SD:0.01 (5.55)
α	Fixed Value	X:0.2115
β	Log normal	X:0.05; SD:0.01 (20)

K01, absorption rate constant; K21, intercompartmental micro-rate constant, α, β, elimination rate macro constants, X, average value; SD, standard deviation; coefficient of variation between brackets.

Table 2. Pharmacokinetic parameters used in the monocompartmental model for fatteners

Parameter	Distribution	Distribution parameters (coefficient of variation%)
Bioavailability (F)	Beta PERT	Min: 0.9; Most likely: 0.95; max:1
Distribution volume (Vd)	Log normal	X:1.4; SD: 0.1(7)
K01	Log normal	X:5.06; SD:1.8 (35.57)
K10	Log normal	X:0.05; SD:0.01 (20)

K01, absorption rate constant; K10, elimination rate constant; X, average value; SD, standard deviation; coefficient of variation between brackets.

Furthermore, the following parameters were calculated to predict the likelihood of developing resistances in fluoroquinolones as described in the literature by Cui *et al.* (2006), Drlica and Zhao (2007) and Zhao and Drlica (2008):

c) PTA in the simulated population of the threshold values $AUC_{0\text{-}24}$/MPC>25 h for each MPC point ranging in geometric progression from 0.002 to 8 μg/mL. Other authors pointed slightly lower $AUC_{0\text{-}24}$/MPC as threshold values (Olofsson *et al.*, 2006; Ni *et al.*, 2013) but it was chosen a value of $AUC_{0\text{-}24}$/MPC>25 h as a worst case scenario.

RESULTS

MPC results

MPC were determined against six strains of App with a MIC of 0.03 μg/mL (n = 3) and 0.06 μg/mL (n = 3). Two strains of Hpp with a MIC of 0.015 and 0.03 μg/mL were also tested. MPC results are shown in Table 3.

For all App strains (MIC of 0.03 to 0.06 μg/mL), the MPC were comprised between 0.12 to 0.5 μg/mL, which corresponded to 2- to 8-fold MIC. No mutants were able to grow at a concentration above 0.5 μg/mL even in strains with reduced susceptibility. In the case of Hp, MPC were equal to 0.015–0.06 μg/mL (1- to 2-fold MIC).

PK simulation

The simulated PK profiles, using the mean, maximum and minimum values obtained from simulations, are presented in Fig. 1. The mean clearance values that were calculated from simulations (Tables 1 and 2) were 0.12±0.11 L/kg/h (coefficient of variation: 91.6%) for fatteners and 0.09±0.02 L/kg/h (coefficient of variation: 22%) for weaners, respectively. These results are in agreement with a clearance value of 0.12±0.02 L/kg/h described by Ding *et al.* (2010). Furthermore, Schneider *et al.* (2014) described clearance values of 0.092 and 0.079 L/kg/h in piglets and fatteners, respectively.

Table 3. Mutation prevention concentration (MPC) and minimum inhibitory concentration (MIC) of six *App* and two *Hp* strains

Isolate details		MIC (μg/mL)	MPC (μg/mL)	
Isolate number	Species	Agar dilution	Duplicate 1	Duplicate 2
1	*A. pleuropneumoniae*	0.03	0.25	0.12
2	*A. pleuropneumoniae*	0.03	0.25	0.12
3	*A. pleuropneumoniae*	0.03	0.12	0.12
4	*A. pleuropneumoniae*	0.06	0.25	0.12
5	*A. pleuropneumoniae*	0.06	0.5	0.12
6	*A. pleuropneumoniae*	0.06	0.25	0.25
7	*H. parasuis*	0.015	0.015	0.03
8	*H. parasuis*	0.03	0.06	0.06

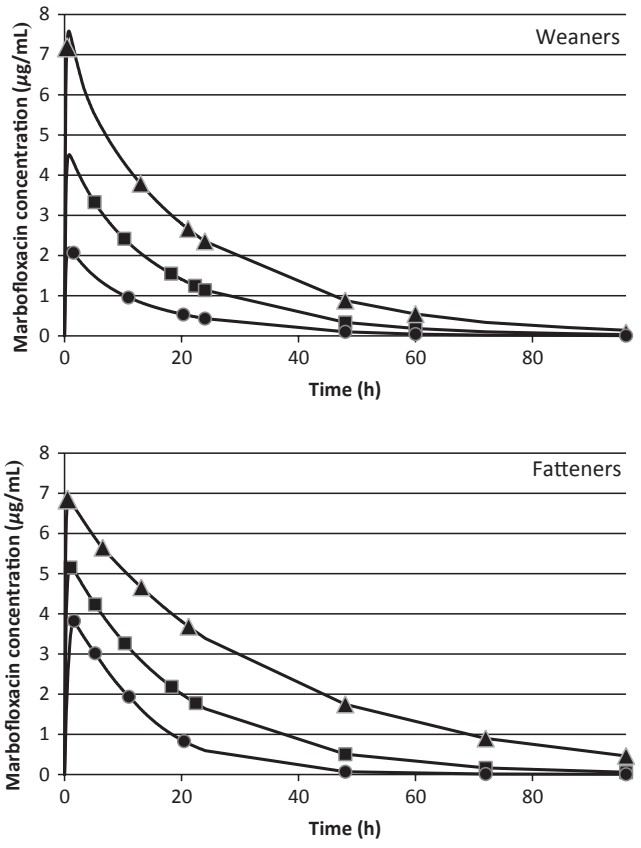


Fig. 1. Pharmacokinetic profiles depicted using the values of the mean (square), maximum (triangle) and minimum (circle) values obtained from the simulations.

Clinical outcome

The probability of target attainment (PTA) of the simulated pig population taking into account a threshold value of $AUC_{0\text{-}24}$/MIC>125 h for a MIC range from 0.002 to 8 μg/mL of App and Hp strains to marbofloxacin is shown in Fig. 2. In this case, the PTA is 100% at the three posology regimen studied for a MIC lower than 0.06 μg/mL for both bacteria (App and Hp) and this value is 0% for MIC values higher than 1 μg/mL in both cases. This PTA value would decrease from 100% to a value lower than 40% in weaner pigs at MIC values of 0.12, 0.25 and 0.5 μg/mL for both bacteria at a dose of 2, 4 and 8 mg/kg bw of marbofloxacin, respectively. In the case of fatteners, the PTA is 100% at the three posology regimen studied for a MIC value lower than 0.12 μg/mL for both bacteria (App and Hp) and 0% for MIC values higher than 1 μg/mL for both pathogens. Finally, the PTA value would decrease from 100% to a value of 0% in fattener pigs at MIC values of 0.25, 0. 5 and 1 μg/mL for both bacteria at a dose of 2, 4 and 8 mg/kg bw of marbofloxacin, respectively (Fig. 2).

The probability of target attainment (PTA) of the simulated pig population taking into account a threshold value of C_{max}/MIC>10 for App and Hp strains to marbofloxacin is shown in Fig. 3. The results obtained are almost the same as previously described (Fig. 2) for the other surrogate marker ($AUC_{0\text{-}24}$/MIC>125 h) with the particularity that the PTA value would decrease from 100% at the same MIC points in fattener (60–80%) than in weaner pigs (20–30%) at 0.12, 0.25 and 0.5 μg/mL for both bacteria at a dose of 2, 4 and 8 mg/kg bw of marbofloxacin, respectively.

The cumulative fraction of responses (CFRs) of the simulated pig population for the three posology regimens according to their probability of MIC strain distribution are shown in Table 4. The same CFRs values were obtained using $AUC_{0\text{-}24}$/MIC or C_{max}/MIC as surrogate markers. The CFR value was higher than 91% and 80.5% for App and Hp for all the studied posology regimens, respectively. Overall, fatteners showed a slightly better theoretical clinical outcome (CFR value) than weaners for both bacteria in all the studied posology regimens reaching the best result at the dose of 8 mg/kg of marbofloxacin in weaners (above 96% for App and 85% for Hp) and fatteners (97% for App and 88% for Hp).

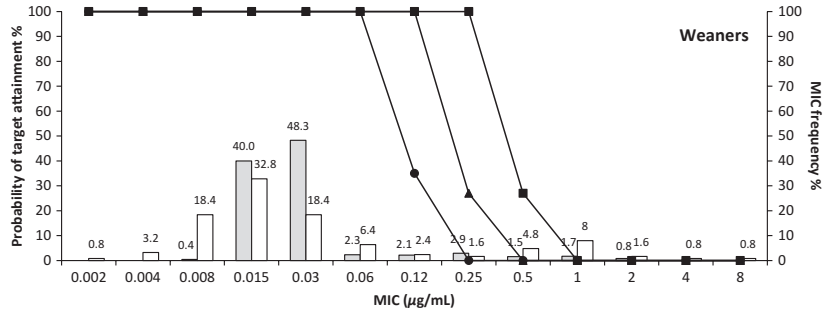
Appearance of resistances

The probability of target attainment (PTA) of the simulated pig population taking into account a threshold value of $AUC_{0\text{-}24}$/MPC>25 h to avoid the appearance of antimicrobial resistance is shown in Fig. 4.

The PTA of the threshold values for preventing antimicrobial resistance of $AUC_{0\text{-}24}$/MPC>25 h across different MPC points (Fig. 4) clearly show that the generation of antimicrobial resistance up to a MPC value of 0.25, 0.5 will be avoided, and 1 μg/mL for both bacteria at a dose of 2, 4 and 8 mg/kg bw of marbofloxacin in weaners, respectively. However, this generation will be probably avoided up to a MPC value of 0.5, 1 and 2 μg/mL for both bacteria at a dose of 2, 4 and and 8 mg/kg bw of marbofloxacin in fatteners, respectively. The same analysis was carried out taking into account the effect of the first dose (data not shown) and it was not observed any difference in comparison with the results obtained for the whole posology regimen.

DISCUSSION

It is widely accepted that drugs are well-designed to cover most of the bug strain population according to PK parameters determined in preclinical studies in the target species. However, it will be very interesting to analyse the probability of success in any antibiotic treatment and in the generation of antimicrobial resistance taking into account the pharmacokinetic and pharmacodynamic variability observed for the pig and micro-organisms, respectively. This type of analysis could be even more relevant if it is taking on board the presence of a population of different strains of the same micro-organism in one animal (Lowe *et al.*, 2012; Vilalta *et al.*, 2012). Finally, this type of analysis could be relevant to foresee the clinical efficacy and the generation of antimicrobial resistance in a dynamic



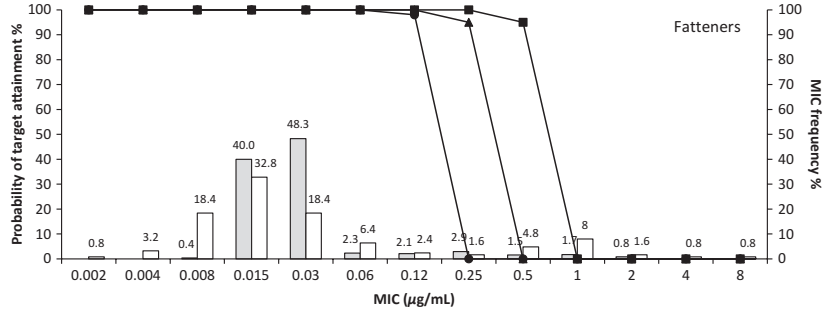
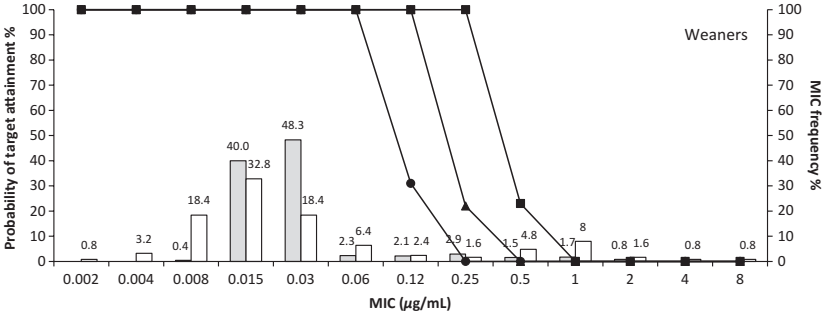


Fig. 2. Graphical representation of the probability of target attainment of the threshold value of $AUC_{0\text{-}24}$/MIC>125 of the simulated values according to each MIC point for weaners and fatteners in the three posology regimens: 2 mg/kg bw (circles), 4 mg/kg bw (triangles) and 8 mg/kg bw (squares) and MIC distribution of marbofloxacin, expressed as strain percentage, against *A. pleuropneumoniae* (grey bars) and *H. parasuis* (white bars).



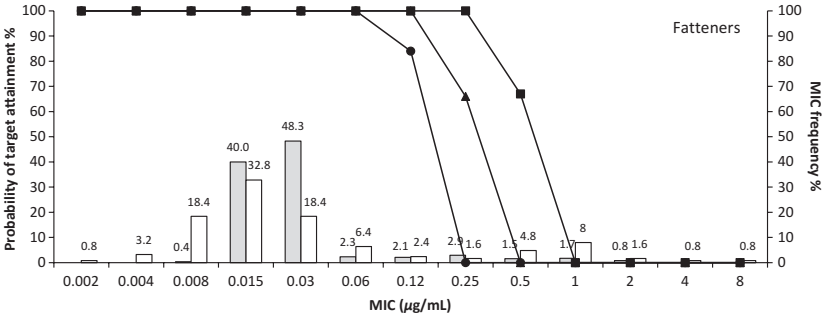


Fig. 3. Graphical representation of the probability of target attainment of the threshold value of C_{max}/MIC>10 of the simulated values according to each MIC point for weaners and fatteners in the three posology regimens: 2 mg/kg bw (circles), 4 mg/kg bw (triangles) and 8 mg/kg bw (squares) and MIC distribution of marbofloxacin, expressed as strain percentage, against *A. pleuropneumoniae* (grey bars) and *H. parasuis* (white bars).

population of micro-organism whose pharmacodynamic properties are continuously evolving.

In this study, PK/PD simulations were performed to evaluate three different posology regimens of marbofloxacin, taking into account the antimicrobial susceptibility of App and Hp and the pharmacokinetic variability of two different groups of pigs, weaners and fatteners. The probability of clinical success was evaluated through the use of the $AUC_{0\text{-}24}$/MIC and C_{max}/MIC index, while the risk of emergence of mutants was evaluated through the use of $AUC_{0\text{-}24}$/MPC index as surrogate markers. Although the same procedures used here have been applied on several occasions to calculate the usefulness of therapeutic strategies in human medicine (Isla *et al.*, 2011; Cao *et al.*, 2013; Goff & Nicolau, 2013), there are some limitations that should be discussed. PK/PD calculations were based on the total drug concentration on serum. It could be assumed that marbofloxacin concentration in the site of action, lung and bronchial secretions, was at least very similar to that observed in serum due to the high bioavailability, low protein binding and tissue distribution reported for fluoroquinolones (Martinez

Table 4. Cumulative fractions of response (CFR) (%) of the threshold values of $AUC_{0\text{-}24}$/MIC>125 h and C_{max}/MIC>10 (between brackets) for the simulated populations of weaners and fatteners when crossed with the MIC distribution probability of the different posology regimens: 2 mg/kg bw, 4 mg/kg bw and 8 mg/kg bw of marbofloxacin

	A. pleuropneumoniae	*H. parasuis*
Weaners		
2 mg/kg bw	91.12 (91.63)	80.52 (80.78)
4 mg/kg bw	93.99 (93.86)	83.19 (82.65)
8 mg/kg bw	96.37 (96.33)	85.1 (85.14)
Fatteners		
2 mg/kg bw	93 (92.92)	82.01 (82.27)
4 mg/kg bw	95.72 (95.08)	83.96 (83.32)
8 mg/kg bw	97.43 (97)	88.49 (87.42)

et al., 2006). Hence, for example, Messenger *et al.* (2012) found that the tissue penetration ratio ($AUC_{tissue}/AUC_{plasma}$) of enrofloxacin in the pleural cavity in pigs was 1.40 ± 0.35 and Bimazubute *et al.* (2009) described a value of 1.26 for the same ratio in the nasal secretions. In conclusion, it seems very reasonable to use the available concentration observed in plasma to foresee the clinical efficacy of this antibiotic. Other clear limitation of this study is that PK parameters used in this research work (from a limited number of animals) could not represent the real interindividual variability of the PK parameters in the targeted animal population not only in healthy animals but also in sick ones. Thus, as an alternative to the previous point, when experimental population data are lacking, the simulations can be performed using '*a priori*' values of the interindividual variability that are 'reasonably' high enough to fit with the real situation. This point has been accomplished in this research work. Thus, the coefficients of variation for marbofloxacin clearance, used during the simulation for fatteners and weaners, were 90 and 20%, respectively. The variability used for this parameter is higher than the previously published by other authors for this molecule (Ding *et al.*, 2010; Schneider *et al.*, 2014). In conclusion, authors believe that the variability used is reasonable enough and it should not be a limitation for the extrapolation of the results obtained to the whole pig population.

This study shows slight differences between the foreseen clinical outcome in late weaners and early fatteners in spite of the differences observed in the pharmacokinetic in both age groups (Schneider *et al.*, 2014) when the effect of the usual posology regimens in use under field conditions (2 mg/kg bw three times each 24 h, 4 mg/kg bw twice each 48 h and 8 mg/kg bw in one single shot) was compared. In this sense, it has to be taken into account that some of the veterinary drugs are not designed to reach a steady-state. Thus, many drugs are designed to get their clinical outcome in one, two or three doses at most with the exemption of the administered orally through water or food. Therefore, it seems that a 'classical' steady-state for many drugs is not reached. An equivalent parameter of the classical AUC/MIC or AUC_{ss}/MIC for those drugs which do not reach the steady-state would be the $AUC_{0\text{-}\infty}$/MIC as pointed out by some authors (Toutain *et al.*, 2007). In this research, the period between 0 and 24 h for the three posology regimes (2, 4 and 8 mg/kg bw) was studied because it seems that marbofloxacin exerts its higher effect on bacteria during this period of time according to an *in vitro* dynamic test carried with strains of *Mannheimia haemolytica* and *Pasteurella multocida* (Vallé *et al.*, 2012). The authors have not found any other information in veterinary medicine comparing different posology regimes using the foreseen effect during the first 24 h. It is clear that further research should be carried out in this matter. On the other hand, other factors besides single or

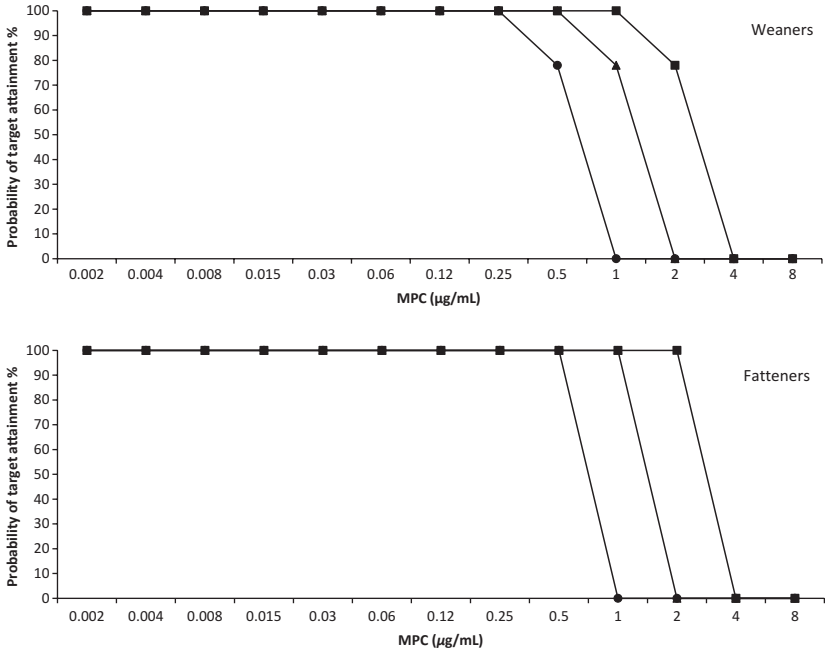


Fig. 4. Probability of target attainment of the threshold value of $AUC_{0\text{-}24}$/MPC>25 for weaners and fatteners in the three posology regimens: 2 mg/kg bw (circles), 4 mg/kg bw (triangles) and 8 mg/kg bw (squares).

multiple doses should be considered when setting the treatment such as the early or late treatment and consequently the size of the bioburden at the site of infection (Ferran *et al.*, 2011) and the MIC or MPC of the offending pathogen. Although, some studies corroborate the efficacy of the multiple dose regimens to eradicate or control a pathogen (Aliabadi & Lees, 2002; Sidhu *et al.*, 2011; Vilalta *et al.*, 2011), the concept of an aggressive early treatment seems to be more suitable to treat infections, reach the PK/PD targets for fluoroquinolones and prevent mutations (Martinez *et al.*, 2012). Our results agree with the literature because the best clinical outcome was foreseen for the 8 mg/kg bw in one single shot. Moreover, similar results were obtained using $AUC_{0\text{-}24}$ or C_{max} as surrogate markers of clinical outcome reinforcing that both parameters could be used for this purpose as it has been published previously in the literature (Drusano *et al.*, 1993; Mckellar *et al.*, 2004; Lees, 2013; Papich, 2014).

Similar values were obtained in the three posology regimens for App and Hp when the effect of preventing resistances was simulated using the marker $AUC_{0\text{-}24}$/MPC. Thus, it would seem quite reasonable to use equally any of the three treatments previously commented. Different opinions can be found in the literature about this topic, while some authors pointed that the single high-dose shot of marbofloxacin would reduce the amplification of resistances (Vallé *et al.*, 2012), other authors concluded that the fractionated dose of the same antimicrobial would be more beneficial to prevent those resistances (Kesteman *et al.*, 2009). Monte Carlo simulations had not taken into account other factors that could lead to the amplification of resistant subpopulations such as the size of the bioburden at the infection site (Ferran *et al.*, 2011), as commented previously, biofilm formation or the effect on other bacterial populations, as the gut flora (Kesteman *et al.*, 2010). Despite these limitations, it is assumed that an early treatment of a highly concentrated drug is more likely to minimize and prevent the amplification of resistances avoiding the growth of the bioburden that could lead to high bacterial density scenario where mutations are more likely to occur. In our case, the 8 mg/kg bw of marbofloxacin in one shot would reach concentrations with a high probability of being above the MPC and would be a reliable option when it comes to prevent the amplification of resistances, at least in the target site. Finally, the results obtained in connection with the generation of antimicrobial resistance should be considered preliminary due to the low number of strains included in the simulation process. This observation is even more relevant in the case of Hp strains where the MPC value is equal or slightly higher than the MIC value. For this reason, additional studies with a higher number of strains are compulsory in order to confirm the obtained results.

Practitioners usually have to start a treatment against App and Hp without knowing the MIC of the causative pathogen. Thus, the CFR calculated in this study could be a good way to estimate the potential for a positive clinical outcome in any herd. To our knowledge, this is one of the first scientific publications in the veterinary field where this approach is carried out. In the future, it could be a good way to select the best antimicrobial to treat an infection following a prudent use of these drugs. It is important to keep in mind that MIC probability distribution of a determined pathogen may vary between countries and regions and even time. Taking into account the MIC distribution provided by Vetoquinol marbofloxacin MIC surveillance program (Giboin *et al.*, 2012), it could be assumed that a marbofloxacin treatment would achieve a CFR of more than 90% (ranging from 91 to 97 depending on the dose) against App and between the range 80–90% against Hp. Although, the CFR for Hp is lower than the App CFR, marbofloxacin would be a reliable option when it comes to treat infections caused by these pathogens. It would have been very interesting to assess CFR for the prevention of resistances, but there is not enough information about MPC and its probability frequencies distribution.

Veterinarians usually treat large populations of animals without knowing the MIC of offending bacteria. Although, the use of CFRs is new in veterinary medicine, it is a used tool in human medicine to compare treatments and foresee clinical failure. Knowing the CFRs of the antimicrobials and bugs should be a good tool to select a treatment and to predict possible outcomes. Thus, PK-PD analysis and Monte Carlo simulations are highly valuable techniques to maximize the favourable result of a therapy but further studies are needed to address this matter.

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IV. DISCUSSION

There are two types of PhD Thesis: perfect and submitted

Anonymous

A recent report of the World Health Organisation (WHO, 2014) on the antimicrobial resistance highlighted that antimicrobials are becoming less effective or ineffective against some infectious agents and remarked the importance of using these drugs in a correct way in both, human and food-producing animals, in order to prevent the spread and development of resistances. This thesis aims to study the effect of MB on two respiratory diseases in swine and how the PK-PD parameters can help to optimise and analyse the effect on the animals.

The first study of this thesis tries to provide information about the penetration of MB in the swine tonsils. The tonsil is where APP can be found in asymptomatic carrier pigs. It would have been interesting to define a more complete time-concentration profile of MB in both, plasma and tonsils, in order to compare the kinetics of the drug in both places like Gehanno and colleagues did with telithromycin in human tonsils (Gehanno *et al*., 2003), where they used three sampling points to depict a primary tonsillar PK profile. Or like Esposito *et al.* (2006) in their paper, where they sampled five time points to describe the PK of moxifloxacin in human tonsils and plasma. However, we could not find any technique that allowed the complete removal of the palatine tonsil without killing the animals and therefore on behalf of animal welfare we had to limit our study. Despite these limitations, our data suggested similarities with other studies focused on the penetration of fluoroquinolones in tonsillar tissue. Thus, Esposito *et al*. (2006) investigated moxifloxacin concentrations in plasma and tonsillar tissue in persons undergoing a tonsillectomy after an oral administration of the drug. In that study average tonsillar concentrations were 2- to 3-fold the average plasma concentrations of moxifloxacin in all five sampling points (2, 3, 6, 12 and 24 hours). Similar tonsillar tissue/plasma concentration ratios were found for ciprofloxacin (1.5-1.9) and levofloxacin (2.02-2.08) after oral or intravenous administrations (Fish & Chow, 1997; Falser *et al.*, 1988). Also similar or lower MB tissue/plasma ratios can be found in pig for other tissues (4 hours after an intramuscular dose of 2 mg/kg body weight), for instance 1.8 at lung, 1.9 at liver, 3.8 at kidney, 1.7 at muscle and 1 at skin level, showing that

tonsil has a similar distribution (ratio tissue/plasma close to 3) pattern when compared to the most studied pig tissues.

In our study I, our hypothesis was that tonsillar tissue will behave as a deep tissue due to the lack of information on antimicrobial or drug penetration in the palatine tonsil in swine. Hurtado *et al.* (2014) studied levofloxacin PK in plasma and prostate tissue in rats. In that study the prostate PK profile fitted better in a three compartment model (deep tissue) and levofloxacin concentrations were a 20 % lower in the prostatic tissue than the respective plasma ones. Comparing the results obtained in the study of Hurtado *et al.* (2014) and the MB distribution ratios commented before, it would seem to point that tonsil PK would fit better in a two compartmental model than in a three one. It is also important to highlight that the result coming from the study of Hurtado and colleagues (2014) came from an interstitial fluid microdyalisis measurement which allows measuring more accurately the drug concentration in the site of action, in that case the interstitial space. On the other hand, drug measurements of our study in tonsils came from a tissue homogenate which can lead to an overestimation of the drug concentration in the target tissue and could imply a wrong interpretation of its clinical efficacy (Mouton *et al.,* 2008). For all the reasons commented above, a more complete tonsillar PK profile is necessary to be described in order to calculate the appropriate PK parameters in this target tissue.

Regarding the MB PK data to calculate the PK-PD parameters used in studies II and III, it was based on the assumption that MB concentration in the lung and nasal mucosa of pig is at least, very similar to the serum concentration. A great deal of information available in the literature supports this idea. FQ, as stated in the introduction, and MB is not an exception, have very good bioavailability, wide tissue distribution and low binding to proteins (Martínez *et al.*, 2006). Furthermore, FQ showed a good tissue penetration ratio ($AUC_{tissue}/AUC_{plasma}$). Thus, enrofloxacin showed a penetration ratio of 1.40 ± 0.35 in the pleural cavity of pigs (Messenger *et al.,* 2012) and a value of 1.26 for the same ratio in nasal secretions in swine (Bimazubute *et al*., 2009). Therefore it seems quite

reasonable to use the plasma concentrations to calculate the PK-PD parameters and foresee their clinical efficacy.

In Study II, PK-PD parameters for efficacy were calculated for the three dosages (2, 4 and 8 mg/kg). Results on PK-PD parameters of Study II-A showed that the only dose of MB with the PK-PD parameters above the threshold associated with efficacy (C_{max}/MIC>8 and AUC_{0-24}/MIC>100) was the 8 mg/kg dose administered in one shot and whose associated PK-PD parameters were C_{max}/MIC=22.4 and AUC_{0-24}/MIC=192. Those results correlated with the results of the PCR technique of HP in the same group where we were not able to detect the microorganism as probably the treatment reduced the bacteria to undetectable levels. However, it is also important to highlight that all the treatments applied were able to reduce significantly the presence of HP in the nasal cavity of pigs when compared to the control group. Furthermore, these results are in agreement with a study investigating the effect of another fluoroquinolones, enrofloxacin, in the *H. parasuis* carrier state in pigs (Macedo *et al.,* 2014) showing a reduction of the amount of bacteria in the nasal cavity of pigs, but not completely eliminating it.

At the end of Study I was concluded that the ratio between the concentration of MB in the tonsil and the MIC_{90} was above the threshold described for clinical efficacy in the doses of 2 mg/kg and 4 mg/kg of MB. Further research using the same procedure described in the material and methods of Study I but with the dose of 8 mg/kg in one shot and the same number of animals (data not published) showed a mean MB concentration of 0.73 µg/mL and 2.27 µg/g in plasma and tonsil respectively, giving as a result a ratio MB_{tissue}/MB_{plasma} of 3 at 24 hours after the MB administration in accordance with the previous results of study I. Using the same MIC_{90} value ,as described in the paper, the ratio MB_{tonsil}/MIC_{90} for this latter dose was 75 suggesting that this last higher dose could also be suitable to eradicate APP from the tonsil. Later microbiological studies carried out on the same farm after that the paper was published showed that the prevalent infectious APP strain of the farm had a MB MIC value of 0.25

µg/mL. When the efficacy ratio was re-calculated again using this farm MIC the MB_{tonsil}/MIC_{farm} ratio changed from 75 to a more realistic ratio of 9 with the dose of 8 mg/kg showing a more reduced PK-PD value but still over the threshold recommended for efficacy. Besides, in this non-published complementary study all the tonsils from the four groups (control, 2 mg/kg, 4 mg/kg and 8 mg/kg) were analyzed using a specific APP PCR technique looking for the presence and viability of APP (Fittipaldi *et al.,* 2003). APP was still found viable in four out of ten tonsils of the 8 mg/kg MB group analysed indicating that APP was not eradicated from the tonsil even though that according to the PK-PD information related to the MB_{tonsil}/MIC ratio it should have been enough MB to kill the bacteria. Some possible explanations to the fact that even reaching the PK-PD thresholds the bacteria was still viable could be:

1. The antimicrobial did not reach the lumen of the tonsil or reached it in a lower concentration than the supposed one. One of the basic principles of PK-PD relationship is that the concentration of a drug should be measured in the site of action relying its activity on the unbound fraction of the same drug (Theuretzbacher, 2007). As stated earlier homogenizing the tissue sample, as in study I, can mislead to believe that antimicrobial is evenly distributed through it. Regarding to FQ, which accumulate intracellularly, homogenising the tissue would lead to an overestimation of the extracellular concentration (Mouton *et al.*, 2008) and consequently to a wrong interpretation of the PK-PD parameters that would be lower than expected. The ideal technique that should be used to measure the unbound portion of the drug in the site of action is the microdialysis (Muller *et al.*, 1998), which allows to have a more realistic measurement of the drug in the interstitial fluid (Hurtado *et al.*, 2014).

2. MB reached the required concentration in the site of action. If this statement would be true then there may be something that may make APP evade the bactericidal effect of MB. Some strains of APP can be able to form biofilm (structured groups of bacteria embedded within a self-produced matrix of extracellular polymeric substance) whether on abiotic or biotic surfaces (Labrie

et al., 2010; Tremblay *et al*., 2013). Even though the colonising APP strains were not able to produce biofilm, they could be embedded in other bacteria biofilm as biofilm can be made up of one or more bacterial species (Mah, T.F. & O'Toole, G.A., 2001). It still has to be elucidated if APP can form biofilm in the pig tonsils but studies in humans found anatomical evidence of bacterial biofilms within the tonsil crypts of patients with recurrent tonsillitis (Chole & Faddis, 2003). Biofilms can resist greater concentrations of antimicrobials (up to 1000-fold) than the same bacteria in a suspension culture (Stewart & Costerton, 2001; Ceri *et al*., 2010). The resistance mechanisms of biofilms still need to be elucidated but some hypothesis have been suggested: slow antimicrobial penetration, altered chemical microenvironment and formation of a resistant phenotype could be the responsible of the high antimicrobial resistance of biofilms (Stewart & Costerton, 2001). Some authors suggested the possibility of using a new type of PD parameter, the minimum biofilm eradication concentration (MBEC) (Olson *et al.,* 2002) to select the correct antimicrobials to treat infections caused by biofilm-forming bacteria. However, an in vitro dynamic model that compared the activities of three fluoroquinolones (marbofloxacin, enrofloxacin and difloxacin) against biofilm-former and biofilm-non-former strains of APP concluded: 1) biofilm-former strains reduced the fluoroquinolone susceptibility in the three FQ and 2) MBEC values were unachievable using a conventional dosage regimen in any of the three FQ (Damte *et al*., 2013).

On the other hand, the combination of two antibiotics or one antibiotic with a biofilm inhibitor has been showed as effective in some *in vitro* cases (Kumon, 2000). After all said above it seems quite reasonable to think that biofilm could be in part responsible of the treatment failure (Mario Jacques, personal communication). However, a lot of information is lacking and further studies should be done in this direction to clarify the MB PK profile in the tonsil and if biofilm contributes to keep the carrier status of APP and to protect it from host defences and antimicrobials.

Another point that should be highlighted is the high MIC found in studies I and II-B. The APP MIC found in the farm of Study I was 0.25 µg/mL. According to the APP MIC distribution data presented in Study III, this high MIC is not usual to be found. Only 3% of the APP strains found in the Vétoquinol Surveillance Program (presented in Study III) have this MIC and only 4 % of the strains found in the same report presented a higher MIC. This high MIC could be the result of a cross-resistance fluoroquinolones phenomenon (Garau, 2000) due to the persistent and repeated use of enrofloxacin in the farm to deal with the APP outbreaks. It would have been ideal to relate this MIC to the probability of target attainment figures of study III (C_{max}/MIC and AUC_{0-24}/MIC) to foresee the population outcome but the lack of information on tonsillar MB PK parameters and their variability makes this difficult to interpret. Similar results were observed in the study II where MICs found in a farm with frequent outbreaks of Glässer's disease, where the use of enrofloxacin was recurrent as well, were in the medium-high part of the HP MIC distribution (study III). In this case it is not as unusual as in the APP case to found HP with high MIC because in the HP MIC strain distribution (study III) the number of strains above the highest MIC found in study II-A (0.25 µg/mL) are 17.6 %. This is in agreement with the information found in the literature where it is described a medium-high level of resistance of HP to fluoroquinolones in Spain and China (Martín de la Fuente *et al*., 2007; Xu *et al*., 2011). Authors do not know whether the high MB MIC found in both farms is a common trait amongst the Spanish swine farms as we could not find any reliable information in the literature on the use of fluoroquinolones in pigs in our country and if this use has lead to a loss of fluoroquinolone susceptibility or if this is a specific and common issue due to the repetitive use of drugs in those farms that have respiratory problems caused by APP and HP.

In this direction, results of Study II-B showed a strain modification towards a less susceptible HP subpopulation after the application of the treatment. Some other papers can be found in the literature describing this phenomenon (Drlica & Zhao, 2007; Roberts *et al*., 2008). However, in our study the effect seemed to be transitory since a diverse and different HP strain population was observed a week after the MB administration. Similar results were observed in *Haemophilus*

influenzae colonization in otitis-prone children where antimicrobial therapy had no relationship with the elimination of the supposed causative strain of *Haemophilus influenzae* and where specific *Haemophilus influenzae* strains reappeared after some months undetected in the nasopharynges (Samuelson *et al*., 1995). Therefore, it seems that antimicrobial treatment does not change the susceptibility of HP in the short term and that the emergence of antibiotic resistance would be the consequence of a more complex interaction of factors involved in the evolution and spread of resistance mechanisms together with a longer and repetitive antimicrobial exposition. In this direction, a 2010 paper published in Nature reveals a non-specific population-based resistance mechanism using indole as a key molecule to develop an increase of the population MIC (Lee *et al*., 2010). This research pointed out that the less susceptible isolates can enhance the survivorship and increase the MIC of the less resistant bacteria in the same population showing that bacteria can have forms of cooperate to overcome the effect of antimicrobials.

In the same Study II-B the potential virulence associated with the virulence-associated trimeric autotransporter (vtaA) was also tested using a specific PCR (Olvera *et al*., 2011) concluding that although antimicrobial treatment selected (temporarily) HP strains with the highest MIC it was not a driving force to select potential virulent strain. This is in accordance with similar findings in *Haemophilus influenzae* (Samuelson *et al*., 1995). Moreover, it was not observed a direct relationship between the presumed virulence and the MIC of the HP strains found in the nasal cavity.

Results of study II-B on how the HP strain population is modified and evolve after the MB treatment raise some interesting question about antibacterial treatments:

1. Is the isolated MIC the highest effective MIC?

Clinicians take the decision to select the antimicrobial and the appropriate posology regimen against a pathogen according to a MIC determined at a laboratory after carrying out the bacterial isolation. In Study II, it was found that different strains with a wide antimicrobial susceptibility can be isolated under field conditions. These results suggest that the isolated strain in diagnostic laboratories could not be the one with the highest antibiotic MIC. This finding might cause an appropriate scenario to develop resistances with common antimicrobial treatments. This observation is extremely relevant and further studies would be necessary to address the variability present in antimicrobial susceptibility for different strains of common bacteria involved in bacterial diseases and analyze it with the usual treatments applied under field conditions.

2. Are the treatments really designed to cover the whole bug strain population?

It is assumed that antimicrobial treatments are designed after clinical studies in the target species having into account the PK of the drug, the PD of the target bug and their PK-PD parameters. However, it would be very interesting to analyse the outcome probability after a treatment or the generation of drug resistance taking into account the PK variability that can be found in different individuals or the or the PD variability (MIC) found in the microbes. This has more relevance if we keep in mind that one animal can be colonized for different strains of the same microorganism as seen in Study II-B or for different members of the same family (Lowe *et al*., 2012) whose PD (MIC) properties are continuously changing. Thus, Study III aimed to evaluate the theoretical outcome of the three most used MB posology regimes against HP and APP taking into account the variability in PK and PD parameters.

In the last study of this thesis we had to face some limitations regarding the MB PK. Firstly, PK calculations and their derivative PK-PD analysis were based on the total drug concentration in serum as explained in the first paragraphs of this discussion. Secondly, the scarcity of PK parameters coming from a limited number of healthy animals could not represent the inter-individual variability of the PK parameters in the targeted population not only in healthy animals but also in sick ones. So, that could be a clear limitation for this study when these results intend to be extrapolated to the whole pig population treated with marbofloxacin. Nevertheless, any new registration procedure for new medicinal products is based on pharmacokinetic studies carried out with a relatively low number of animals (8-10) and there is a scarce of information in the literature about variation in pharmacokinetic parameters based on a large animal population. Thus, authors have carried out this study with the all the available information provided by the marketing authorization holder for marbofloxacin (Vétoquinol SA). To overcome the limitation and when population data are lacking, simulations can be performed using theoretical values that are reasonably high enough to represent the real situation. For instance, coefficients of variation of clearance higher than 50% are classically observed in the literature. After revising in detail the raw data, the coefficients of variation for marbofloxacin clearance used during the simulation for fatteners and weaners was 90 and 20%, respectively. The variability used for this pharmacokinetic parameter in this research work is higher than the previously published by other authors for this molecule (Ding *et al.*, 2010; Schneider *et al.,* 2014). Thus, authors believe that the variability used is reasonable enough and it should not be a limitation for the extrapolation of the results obtained to the whole pig population.

The later study gives a prediction of the clinical outcome taking into account the effect of MB in pigs on the APP or HP strain population during the first 24 h showing little differences between the weaners and fatteners. These differences between the foreseen outcome in late weaners and early fatteners could be attributed to the PK differences found in the different age groups where weaners have a bigger clearance than fatteners, 0.092 and 0.079 L/h·kg

respectively (Schneider *et al*., 2014). Clearance age-related changes has been described in humans (Rowland & Tozer, 2011) and consequently different dosage regimes have been assessed for fluoroquinolones for elderly patients (Leroy et *al.*, 2012) or for critically ill patients whose clearance could be impaired (Khachman *et al.*, 2011). On the other hand, this parameter increases for marbofloxacin as age increases in goats and other ruminants due to some physiological and anatomical changes (Waxman *et al*., 2004). Regarding this, it would be important to know the age pharmacokinetic specificities of the group to treat as it could lead to a treatment failure.

MB and many other veterinary drugs are not designed to reach a classical steady state as in humans where medication can be administered by perfusion or during long medications. Thus, the veterinary antimicrobials are intended for doing their effect in one, two or three doses with the exceptions of those drugs that can be administered orally (via feed or water) or by perfusion (usually pets). Therefore it could seem that the parameter AUC_{ss}/MIC is not suitable in this case since the steady state is not reached. However, some authors stated that an equivalent parameter for these drugs that does not reach the steady state could be $AUC_{0-\infty}$/MIC (Toutain *et al.,* 2007). We decided to simulate the effect of the first 24 h for the three posology regimes as it seems that MB has a higher activity on microbes during this period as published by Vallé and colleagues in an *in vitro* dynamic test with bovine respiratory pathogens (Vallé *et al.*, 2012).

It would have been also very interesting to evaluate the differences between the different treatments but the lack of an appropriate tool or parameter that allows us to compare different treatments makes this comparison very difficult. However, and looking at the results of Study II, the 8mg/kg bw MB in one shot seemed the best option. Other factors besides single or multiple doses application should be taken into account when treating an individual or a whole population. These other factors are: the early o late application of the treatment, the size of the bacterial burden at the infection site (Ferran *et al*., 2011) or the MIC and MPC of the infectious agent. In the literature some studies can be

found that corroborate the efficacy of a multiple dose regimen to control or eradicate bacteria (Aliabadi & Lees, 2002; Sidhu *et al.,* 2011). However, the concept of a more aggressive early treatment that reaches a quick effective antimicrobial concentration in the site of action and consequently an early attainment of the PK-PD target for fluoroquinolones is desired (Martinez *et al*., 2012). This is in agreement with the foreseen clinical outcome obtained in Study III having its best effect for the dose of 8 mg/kg. Results of study IIA corroborate the previous points being the highest dose the only one that reduced the amount of bacteria in the nasal cavity to undetectable levels. Furthermore, similar results were found when simulating the foreseen clinical efficacy with both surrogate markers for fluoroquinolones, $AUC_{0\text{-}24}$ and C_{max}, reinforcing the idea that both parameters can be used to predict the clinical efficacy outcome (Drusano *et al.,* 1993; Mckellar *et al*., 2004; Lees, 2013; Papich, 2014).

In another part of Study III we simulated the rate of attainment of the parameter for preventing resistances $AUC_{0\text{-}24}$/MPC finding similar results between posology regimes and between fatteners and weaners. In this simulation it was not taken into account the effect of single or multiple drug administrations. Although the $T_{>MPC}$ / $T_{MSW,}$ parameter described for Kesteman *et al.* (2009), would be the best suggested parameter to compare single or multiple administrations the lack of an indicative cut-off value and the scarcity of information on how this parameter is related to the appearance of resistances makes it difficult to use and interpret. Regarding the use of single or multiple doses to avoid the appearance of resistances different points of view can be found in the existent literature. Thus, Vallé *et al.* (2012) suggested that the use of a single high dose of 10 mg/kg of MB is preferred to reduce the amplification of resistances in front of a multiple dose administration of 2 mg/kg of MB in bovine pathogens. On the other hand, the research of Kesteman *et al*. (2009) supports the idea that a fractionated dose would exert a bigger effect on the resistant subpopulations than the same dose administered in one shot. Simulations only take into account the PK and PD parameters that are introduced in the simulation program and do not take into account other agents that could be involved in the appearance and increase of resistance such as the

bioburden size at the target site (Ferran *et al.,* 2011), the effect of biofilm or the effect that the antimicrobial could have on other bacterial populations on the same individual, e.g. the gut flora (Kesteman *et al*., 2010). However, it seems that an early application of a treatment would be beneficial reducing the probabilities of appearance of resistance through avoiding the growth of the bioburden to a scenario where resistances are more likely to occur. It is worth to remind that bacterial mutation frequency has been hypothesized to be between 10^{-6} and 10^{-10} (Martinez *et al.*, 2012). Purulent fluids may contain an average bacterial cell counts of $2x10^8$ CFU/ml or higher in some cases (10^9 CFU/ml) in humans (König *et al.,* 1998). Besides it seems that the bacterial population would be large enough to contain resistant mutants by the time the patient shows clinical signs after a bacterial infection (Drusano, 2004). Therefore, considering that in our study the 8 mg/kg MB in one shot would reach concentrations with a high probability of being above the MPC in the target site it seems that this posology regimen would be a good option in avoiding the amplification of resistances, at least in the infection site studied. Finally, this data should be considered preliminary due to the scarcity of information on MPC strain distribution and additional studies are recommended in order to confirm these results.

Selecting a breakpoint is a process that integrates microbiological, PK-PD and clinical data. One input that has to be taken into account when a breakpoint has to be set is the information coming from PK-PD modeling and Monte Carlo simulations (EUCAST). When setting PK-PD breakpoints the CLSI set them at the last highest value of MIC that reaches at least 90% of the PTA (Maglio *et al*., 2005). If we compare the existing susceptibility breakpoints for MB in the literature for dogs and cats (CLSI, 2007) and for the following bacterial groups: Enterobacteriaceae, Pasteurellaceae, Staphylococcus spp. and Streptococcus spp. (CASFM, 2010) with the PK-PD breakpoints resulting from figure 2 of study III (PTA of $AUC_{0\text{-}24}$/MIC>125) it is worth noticing that existed a little discrepancy. Whilst literature breakpoints are set in S≤1 µg/mL and R>2 µg/mL, PK-PD breakpoint coming from the simulations ranged from 0.06 µg/mL to 0.25 µg/mL in weaners and from 0.12 µg/mL to 0.5 µg/mL in fatteners for the 2, 4 and 8

mg/kg doses respectively. In addition, Vétoquinol recommended breakpoints are equally set in S≤1 µg/mL and R>2 µg/mL relating these to the diameter of inhibition after being validated on strains coming from cats, dogs, cattle and pigs. PK-PD breakpoints are related to dose, the higher the dose the higher the PK-PD breakpoint and theoretically a better clinical outcome because more animals will reach the suggested PK-PD threshold (AUC/MIC) for clinical efficacy. In our study PK-PD breakpoints are lower in 1, 2, 3 or even 4 MIC dilutions depending on the dose and the age group than the breakpoints suggested by CASFM or CLSI. These findings are in agreement with previous studies in human infections that pointed out discrepancies between PK-PD, CLSI and EUCAST breakpoints in Gram-negative (Frei *et al*., 2008) and Gram-positive bacteria (Asin *et al*., 2012) showing that when the PK-PD breakpoints were considered those tended to be lower than the ones defined by EUCAST or CLSI. On the other hand, in a recent simulation study where amoxicillin breakpoints were compared with those cut-off values extracted from Monte Carlo simulations of different dosage regimes, it was established that cut-offs are dependent on dose and route of administration as only the highest simulated oral dose reached the suggested PTA values (higher than 90%) at the established breakpoint of the CLSI for amoxicillin of 0.5 µg/mL. Furthermore only the 54% of the simulated profiles of the highest IM dose (30 mg/kg) reached the threshold for efficacy for amoxicillin at the suggested breakpoint (Rey *et al*., 2014). The establishment of cut-off values using Monte Carlo simulations have some limitations that must be highlighted: 1) PK data used in the simulations usually comes from healthy animals with no affected distribution and elimination 2) Models are just equations that facilitates the understanding the calculation of drug exposure 3) PK data is usually obtained from serum sampling. Therefore, depending on the PK behavior of a drug the result of the simulations may not be applicable to other tissues. Another point that we should keep in mind is that antimicrobial therapy by itself does not “cure” the animal, immunity also plays an important role. Setting a breakpoint takes into account not only PK-PD data and Monte Carlo simulations but also microbiological (MIC distribution), clinical and pharmacological (PK) data. In addition, most of the veterinary breakpoints in use come from extrapolated data from human medicine. Although it is not the MB case as MB it is only used in veterinary

medicine. During the last years, the CLSI subcommittee for Veterinary Antimicrobial Susceptibility Testing (VAST) has been working to expand the drugs list on which there are veterinary-specific breakpoints (CLSI, 2013; Papich, 2014). All in all, more information is needed about “real” veterinary breakpoints in order to use the antimicrobials in a more effective way and not to rely on those breakpoints coming from human medicine or extrapolated from other species that could lead to an inappropriate use of antimicrobials.

Frequently when clinicians have to start a treatment against APP and HP the MIC of the causative pathogen is unknown. So, to calculate the CFR would be a good way to estimate the potential for a clinical outcome of the infected herd when no other information was available. Thus, regarding this parameter, it is worth knowing that a CFR≥90% is considered optimal against a bacterial population, whereas a CFR≥80% but ≤90% is associated with moderate probabilities of success (Bradley *et al*., 2003). In addition, it is important to keep in mind that MIC probability distribution of a determined pathogen may vary between countries and regions and even time. Thus, for example the results on marbofloxacin resistance of APP in Italy published by Vanni *et al.* (2012) showed a percentage of resistance that went from 16.7% in 2000 to 2% in 2009 being this latter value very similar to the percentage marbofloxacin resistant strains observed by Vétoquinol. Taking into account the MIC distribution provided by Vétoquinol marbofloxacin MIC surveillance program (published by Giboin *et al*., 2012) it could be assumed that a marbofloxacin treatment would achieve a CFR of more than 90 % (ranging from 91 to 97 depending on the dose) against APP and between the range 80-90% against HP (ranging from 80 to 88 depending on the dose). Although, the CFR for HP is lower than the APP CFR, marbofloxacin would be a reliable option when it comes to treat infections caused by these pathogens. It would have been interesting to calculate the CFR for the appearance of resistances but, as commented previously in another paragraph, the lack of information on how MPC distributes would lead to a poor estimation of this parameter. In summary, CFR would be a very practical parameter for practitioners because it gathers the PK information of the population to be treated and the MIC strain distribution of the offending

pathogen showing the potential estimation of a positive clinical outcome in the herd. Hence, if practitioners had the CFR of different antimicrobials and different microorganisms they could select the best ‘a priori’ option to treat the herd whilst waiting for more accurate MIC diagnose. However, further studies are needed to be carried out to expand the knowledge about this subject.

Overall, this thesis reinforces the idea of considering not only the antimicrobial activity of the fluoroquinolone MB but also the dosing regimen to increase the probability of clinical success of the antimicrobial treatment in front of two respiratory diseases in swine. However, some points need to be further studied in order to prevent the misuse of antimicrobials and expand the knowledge of their relationship with bacteria:

-PK profile of MB in the tonsil.

-Role of biofilm in APP disease maintenance in the herd.

-Effect of the decrease in nasal carriage of HP on the spread of the disease within the herd.

-Real MB MIC strain distribution of APP and HP in Spain.

-Study the real variability of MB PK parameters through an extensive population PK analysis.

-Set real veterinary breakpoints that are bug and drug specific not only for MB but also for all the veterinary antimicrobials.

-Explore the potential use of CFR in predicting the favorable outcome of a treatment.

V. CONCLUSIONS

The more original a discovery the more obvious it seems afterwards

Arthur Koestler

1. Marbofloxacin is detected in tonsils at 24 hours after the administration of different posology regimes in a dose-dependent fashion. MB can be found three times more concentrated in the tonsil than in the plasma at 24 hours post-administration.

2. It was still detected viable bacteria at the tonsil at the dose of 8 mg/kg even with a MB tonsil concentration/MIC_{farm} above the threshold values for clinical efficacy.

3. The administration of marbofloxacin decreases the prevalence of HP at nasal mucosa after administering marbofloxacin at 2, 4 or 8 mg/kg. Furthermore, the bacteria were not detected 24 hours after the application of the highest MB dose.

4. The antimicrobial treatment selects temporary HP strains with highest MIC but it does not mean that this treatment is a driving force to select virulent HP strains.

5. PK-PD breakpoints for clinical efficacy extracted from the simulation ranged from 0.06 µg/mL to 0.25 µg/mL in weaners and from 0.12 µg/mL to 0.5 µg/mL in fatteners for the 2, 4 and 8 mg/kg doses respectively. Besides, the results are the same for both PK-PD parameters, the $AUC_{0\text{-}24}$/MIC and the C_{max}/MIC.

6. CFR of MB against APP shows high probabilities of success for the three doses in both, weaners and fatteners, with percentages over 90%. However, the same parameter only showed moderate possibilities of success when MB is used against HP, no matter which age group is treated, with CFR ranging from 80 to 88% depending on the dose.

7. Taking into account the PTA of the threshold value $AUC_{0\text{-}24}$/MPC>25 to avoid the appearance of antimicrobial resistance, PK-PD breakpoints would range from 0.25 µg/mL to 1 µg/mL in weaners and from 0.5 µg/mL to 2 µg/mL in fatteners for the 2, 4 and 8 mg/kg doses respectively.

VI. ANNEX

All things are difficult before they are easy

Thomas Fuller

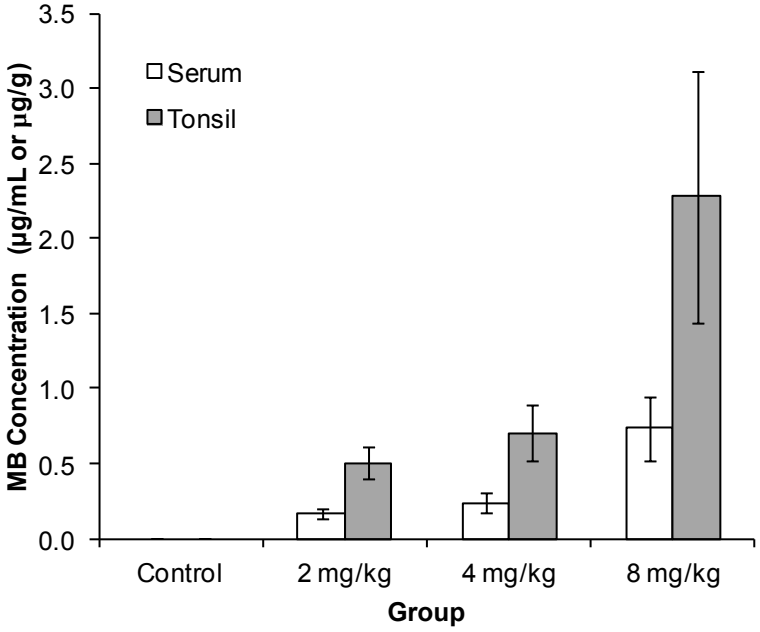

Figure 1. Mean concentration (± standard deviation) of MB in serum (white bars) and tonsils (grey bars) 24 h after the last intramuscular administration of MB at 2, 4 and 8 mg/kg administered three times (every 24 h), twice (with a 48 h interval) and single shot, respectively, in 10 pigs for each experimental group.

VII. REFERENCES

An author is a fool who, not content with boring those he lives with, insists on boring future generations.

Charles de Montesquieu

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