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Challenging the prescribed enrofloxacin treatment regimen of bacterial respiratory disease caused by *Ornithobacterium rhinotracheale* and *Escherichia coli* in turkeys

An Garmyn

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TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

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APEC	avian pathogenic *Escherichia coli*
APV	avian metapneumovirus
AUC	area under the curve
BHI	brain heart infusion
BW	body weight
C_{lb}	body clearance
C_{max}	maximum serum concentration
CD_{50}	ciliostatic dose
cfu	colony forming units
CO_2	carbon dioxide
DNA	desoxyribonucleic acid
E5-5D	treatment group receiving 5 mg enrofloxacin/kg bodyweight for 5 days
E10-5D	treatment group receiving 10 mg enrofloxacin/kg bodyweight for 5 days
E15-3D	treatment group receiving 15 mg enrofloxacin/kg bodyweight for 3 days alternated each time by a non treating day
E20-5D	treatment group receiving 20 mg enrofloxacin/kg bodyweight for 5 days
E25-2D	treatment group receiving 25 mg enrofloxacin/kg bodyweight for 2 days alternated by a non treating day
E50-5H	treatment group receiving 50 mg enrofloxacin/kg bodyweight for 5 hours
E50-10H	treatment group receiving 50 mg enrofloxacin/kg bodyweight for 10 hours
E50-20H	treatment group receiving 50 mg enrofloxacin/kg bodyweight for 20 hours
ELISA	enzyme-linked immunosorbent assay
h	hour
HEPA	high efficiency particulate air
kg	kilogram
l	liter
LPS	lipopoly-saccharides
M	molar
MBC	minimal bactericidal concentration
mg	milligram

MIC_{50}	minimum inhibitory concentration required to inhibit the growth of 50% of the organisms
MIC_{90}	minimum inhibitory concentration required to inhibit the growth of 90% of the organisms
ml	milliliter
MPC	mutant prevention concentration
MRT	mean residence time
NC	negative control group
Omp	outer membrane protein
P	P-value
PAE	post-antibiotic effect
PALE	post-antibiotic leukocyte enhancement effect
pbi	post bacterial infection
PBS	phosphated buffered saline
Pc	partition coefficient
PC	positive control group
PCR	polymerase chain reaction
pKa	acid dissociation constant
ppm	parts per million
pvi	post viral infection
Qnr gene	quinolone resistance gene
QRDR	quinolone resistance-determining region
RNA	ribonucleic acid
rpm	rotates per minute
SM-PAE	subMIC- post-antibiotic effect
SPF	specified pathogen free
$t_{1/2}$	elimination half life
TNFα	tumor necrosis factor α
TOC	tracheal organ culture
µg	microgram
µl	microliter

CHAPTER 1: GENERAL INTRODUCTION

1.1. ENROFLOXACIN, A FLUOROQUINOLONE COMPOUND

1.2. MULTICAUSAL RESPIRATORY DISEASE IN TURKEYS

GENERAL INTRODUCTION

1.1. ENROFLOXACIN, A FLUOROQUINOLONE ANTIMICROBIAL COMPOUND

1.1.1. General considerations of enrofloxacin use in turkeys

In modern poultry industry, turkeys have been selected for growth rate and feed efficiency rather than for robustness and resistance to disease. Disease outbreaks often lead to extensive carcass condemnations at processing, resulting in significant economic losses. To minimize these economic losses due to microbial disease, therapeutic programs are needed using agents that have the ability to rapidly kill any bacterial pathogen that invades the poultry flocks (Tanner, 2000). Other important matters to consider are the cost-to-benefit ratio of the treatment, the available administration route, the drug withdrawal time, and the antimicrobial susceptibility of the target pathogen (Tanner, 2000).

After flumequine, enrofloxacin was the second antimicrobial fluoroquinolone compound used in veterinary medicine. The drug is active against pathogenic bacteria that play a significant role in turkey respiratory disease or septicaemia: *Mycoplasma* sp., *Avibacterium paragallinarum*, *Pasteurella* sp., *Escherichia coli*, *Staphylococcus sp.* and *Erysipelotrix rhusiopathiae.* The current treatment schedule advises administration of enrofloxacin at a dose of 10 mg/kg body weight for 3 to 5 continuous days. When mixed bacterial infections are involved treatment should last for 5 continuous days. After such treatment several clinical trials have observed a rapid decrease in mortality, clinical symptoms and respiratory tract lesions. In most cases, a complete eradication of the causative bacterial organism was reported (Braunius, 1987; Behr *et al*., 1988; Hafez *et al*., 1992; Cargill, 1995; Gautrais and Copeland, 1997; Marien *et al.*, 2007). When involved in the infection, complete eradication of *Mycoplasma* sp. was not accomplished, but enrofloxacin treatment reduced the mycoplasma excretion and mortality rates (Hinz and Rottmann, 1990; Rainhardt *et al.*, 2005).

Enrofloxacin can be administered through drinking water. This route of administration proves most effective in clinical outbreaks of microbial disease (Tanner, 2000). Advantages of water medication are the rapid response, medication reaching the birds within short time and convenience of administration. Possible disadvantages are that impurities in the water (bi- or trivalent cations) may reduce absorption. The acrid taste of enrofloxacin which may contribute to variations in water consumption in pigs, does not affect the water palatability in poultry. Parenteral injection delivers a correct dose but is labour-intensive and results in

considerable stress in the birds. Feed incorporation of antimicrobials for therapeutic purpose is less effective because of the time to manufacture, the inappetence and the inability to compete for feed in sick animals. Antimicrobial sensitivity testing should be conducted to support the antimicrobial treatment and should be repeated if no clinical improvement is noticed after two or three days of treatment.

1.1.2. Structure/activity relationships and physicochemical properties of enrofloxacin

Enrofloxacin is a fluoroquinolone antimicrobial drug derived from nalidixic acid. Like all quinolones, it is manufactured synthetically. Nalidixic acid, discovered in 1962, was the first clinically approved 4-quinolone-type compound, but had several limitations. The drug showed only modest activity against Gram-negative bacteria, restricted to the *Enterobacteriaceae*, and possessed poor pharmacokinetic properties regarding absorption and distribution. Therefore, nalidixic acid proved only effective against urinary tract infections. In addition, the molecule had a tendency to select for resistant organisms and induced several toxic effects (Appelbaum and Hunter, 2000; Walker, 2000b; Martinez *et al.*, 2006). Hence, several structural modifications have been implemented to the "original" molecule of nalidixic acid, to improve its antibacterial activity and its pharmacokinetic features (Chu and Fernandes, 1989; Sarkozy, 2001).

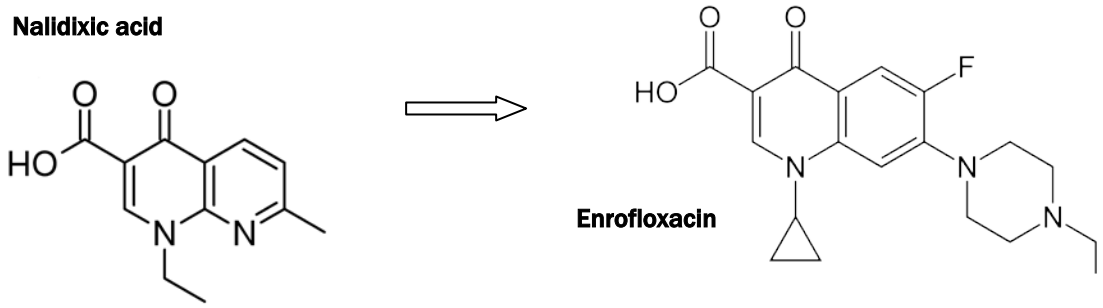

Figure 1. Structural formulas of nalidixic acid and enrofloxacin (adapted from Sarkozy, 2001).

First, the naphthyridone nucleus (N at position 1 and 8) was transformed into a quinolone nucleus, a ring structure containing only one nitrogen in position 1. Halogenating position 6 of the quinolone nucleus with a fluorine atom substantially broadened the antibacterial activity spectrum by improving the clinical activity against Gram-positive bacteria and enhanced oral bioavailibility and tissue penetration (Chu and Fernandes; 1989; Sarkozy,

2001). The introduction of the piperazinyl (a heterocyclic nitrogen-containing ring) side chain at position 7 of the quinolone nucleus enhanced activity by increasing the ability of the drug to penetrate the bacterial cell wall improving once more activity against Gram-negatives, including *Pseudomonas* sp. (Chu and Fernandes; 1989; Sarkozy, 2001). Adding a cyclopropyl-group at position 1 of the nitrogen ring and an etylgroup to the piperazinyl ring increased the lipid solubility and the volume of distribution of the compounds (Sarkozy, 2001) again enhancing potency against members of the family *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Chu and Fernandes, 1989).

All these transformations lead to the construction of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-ethyl-1-piperazinyl)-3-quinolone-carboxilic acid, or enrofloxacin, with the following physicochemical properties. The dissociation constants of enrofloxacin are pKa1=5.94 and pKa2=8.70 corresponding to the carboxylic acid group on the 3- position and to the basic piperazynil group in the 7-position, respectively (Lizondo *et al.*, 1997). These constants help define the state of ionization of enrofloxacin since the molecule can exist in four possible analogues depending on the environmental pH: an acidic cation, a neutral un-ionized species, an intermediate zwitterion and a basic ion (Lizondo *et al.*, 1997). Maximal transfer of enrofloxacin from the aqueous to the lipid phase occurs at pH 7.0. Below and above this pH, partition coefficients (Pc) of the drug decrease (i.e. Pc<3.48) indicating higher polarity and, therefore, minor transfer (Lizondo *et al.*, 1997; Martinez *et al.*, 2006). This lipophility of enrofloxacin facilitates its diffusion into biological tissues, including bacterial cells. The aqueous solubility of enrofloxacin is low between pH 6.0-8.0, which is near its isoelectric point. Maximal aqueous solubility (i.e. 10.42 mg/ml) is achieved at pH 5.02. When a concentrated acetate buffer is used, the amount of enrofloxacin solubilized can be increased (> 100 mg enrofloxacin/ml using a 1.178 M acetate buffer) (Lizondo *et al.*, 1997). According to these physicochemical properties with moieties of the molecule being lipophilic and lipophobic as well as having different areas of positive and negative charge (zwitterion properties) enrofloxacin has the capacity to cross biological membranes easily and achieve high concentrations in body tissues.

1.1.3. Bacterial topoisomerases and the mechanisms of enrofloxacin action

The bacterial chromosome is a continuous, circular, double-stranded DNA molecule approximately 1.000 times longer than the bacteria in which it is contained. To fit into the cell, the DNA ring must be condensed into a negative supercoil through repeated twisting (Walker, 2000b; Hawkey, 2003). Each one of such twists results in one superhelical turn,

which can only be released by breaking the covalent bonds of the DNA backbone. Enzymes that catalyze such conversions are called topoisomerases (Messer, 1999; Walker 2000b; Hawkey, 2003). Enrofloxacin inhibits the activity of two of those enzymes: DNA gyrase and topoisomerase IV (Hawkey, 2003).

DNA gyrase (or topoisomerase II) is a tetramer of two A and B subunits A2B2, encoded by the *gyrA* and *gyrB* genes, respectively (Hawkey, 2003; Martinez *et al.*, 2006). The enzyme introduces negative supercoils into DNA, which is important for initiation of DNA replication, and relieves torsional stress expected to accumulate ahead of transcription and replication complexes (Drlica, 1999; Hooper, 2000; Hawkey, 2003). This is obtained by wrapping DNA into a positive supercoil and then passing one region of duplex DNA through another via DNA breakage and rejoining (Hawkey, 2003). In addition, the enzyme removes knots from the DNA and helps bending and folding it (Drlica and Zhao, 1997). DNA gyrase is the primary target of enrofloxacin for Gram-negative bacteria (Hawkey, 2003).

Composed of two C and E subunits and encoded by the *parC* and *parE* genes respectively, topoisomerase IV is similarly structured as DNA gyrase. Also the reaction mechanism is similar to that of DNA gyrase, although topoisomerase IV binds to DNA crossovers rather than wrapping DNA. The enzyme is primarily involved in decatenation, the unlinking of daughter chromosomes following replication so that segregation into the daughter cells can occur (Hawky, 2003). Topoisomerase IV is the primary target of enrofloxacin in Gram-positive bacteria (Hawkey, 2003).

Enrofloxacin induces a conformational change in these two topoisomerases which traps them on the bacterial DNA as drug/enzyme/DNA-complexes. This prevents religation of the broken DNA strands and thus DNA synthesis and cell growth (Drlica, 1999).The bacteriostatic action of enrofloxacin is related to this complex formation. The bactericidal action of enrofloxacin, however, is thought to be a separate event and three modes of cell killing are suggested (Brown and Reeves, 1997; Martinez *et al.*, 2006).

The first mode involves removal of gyrase-drug complexes from DNA and liberation of lethal double strand DNA breaks. This mechanism requires RNA and protein synthesis and is only effective against dividing bacteria. The second mode of action postulates that DNA ends are released, albeit with the gyrase subunits attached. This mechanism does not require RNA and protein synthesis (since it is chloramphenicol insensitive), can act on bacteria that are unable to multiply and becomes more prominent as drug concentrations increase. The third mode requires RNA and protein synthesis but does not require cell division. This mechanism may correlate with trapping of topoisomerase IV complexes on DNA.

Some aspects of bactericidal action fail to fit into these schemes. High concentrations of the drug (quinolones), for example, are not as effective in killing bacteria as moderate concentrations (Drlica and Zhao, 1997). It has been suggested that inhibition of RNA synthesis, which occurs at high concentrations might interfere with lethal removal of complexes. However, at high concentrations, ciprofloxacin, a metabolite of enrofloxacin, predominantly kills by a mode that is not blocked by protein synthesis (Drlica and Zhao, 1997). A satisfactory explanation for this behaviour has not yet been found and therefore the precise mode of action of the drug is not yet completely understood.

Additionally, enrofloxacin exhibits immunomodulatory effects. *In vitro*, it stimulates the oxidative capacity of phagocytic neutrophils (Hoeben *et al.*, 1997a; 1997b) enhancing their phagocytic killing and therefore strengthening the innate host defense. Ciprofloxacin, a metabolite of enrofloxacin, downregulates the release of cytokines such as TNFα (Khan *et al*., 2000; Purswani *et al.*, 2002) reducing the acute phase response which is a cascade of harmful inflammatory reactions caused by exposure to bacterial endotoxin (also known as LPS compound of the Gram-negative cell wall, released in the blood stream during bacteraemia or in inflammatory tissue).

1.1.4. Acquired resistance to enrofloxacin

Acquired resistance to fluoroquinolone antimicrobials, including enrofloxacin, is most often chromosomally mediated. The major mechanisms are mutations in the drug targets, over-expression of efflux pumps and loss of porins (Brown and Reeves, 1997; Hooper, 2001a, 2001b; Chen and Lo, 2003). After acquirement the organism passes the resistance mechanism vertically to surviving progeny (Robicsek *et al*., 2006a).

Mutations in the drug targets are the primary mechanism for enrofloxacin resistance which lead to inefficient binding of enrofloxacin to the target enzymes. In Gram-negative bacteria (e.g. *E. coli*) the primary target (for the first step mutations) is the *gyr*A gene of the DNA gyrase (Hooper, 2001a). Mutations in the *gyr*A gene are located in a small region called the quinolone resistance-determining region (QRDR). Two amino acids most commonly substituted are Ser83 and Asp87 (Hooper, 2001a). Recently, substitution of the same amino acids after mutation in the *gyr* A gene have been reported in *O. rhinotracheale*, resulting in isolates with reduced susceptibility against enrofloxacin (Marien *et al.*, 2006a). In some Gram-positive bacteria (e.g. *S. aureus*) the primary target is the *parC* gene of topoisomerase IV (Ferrero *et al*., 1994; Hooper, 2001a). For this *parC* gene a similar QRDR has been

reported. Mutations in the *gyrB* and *parE* genes are less common (Chen and Lo, 2003; Hooper, 2001a).

In most bacteria, first step mutations lead to only small (2 to 8) increases in minimal inhibitory concentrations (MICs). The organism becomes less susceptible to enrofloxacin, a condition that is not clinically detectable. Only after accumulation of additional point mutations in different segments of the bacterial genome encoding for the topoisomerases resistance is conferred. Single-step mutation frequencies may vary among bacterial species. In *E. coli* the single step mutation frequency is $<10^{-7}$ (Lindgren *et al.*, 2005), in *Mycobacterium* sp. 10^{-7} to 10^{-8} (Sindelar, 2000) and in *Chlamydia* sp. 10^{-8} (Rupp *et al.*, 2008). Consequently, frequencies at which bacteria develop resistance to a fluoroquinolone compound (i.e. after two concurrent, independent target mutations) are low and rated at 10^{-14} to 10^{-16} (Drlica, 2003; Strahilevitz and Hooper, 2005). Resistance after single step mutations is only seen in *Campylobacter* sp. (Payot *et al.*, 2002) and in species with MICs already high like *Pseudomonas* sp. *and Staphylococcus* sp. (Brown and Reeves, 1997). However, when resistance to one quinolone is acquired, at least some degree of resistance to all the other drugs in this group has been observed (Brown and Reeves, 1997).

Increasing the activity of efflux pumps does not confer but may contribute to resistance to fluoroquinolones (Brown and Reeves, 1997; Hooper, 2001a; Chen and Lo, 2003)**.** These efflux systems are typically capable of causing resistance to non-structure-related antimicrobials and are referred to as multi drug resistant pumps (Hooper, 2001a). Efflux pump systems leading to fluoroquinolone resistance identified in Gram-negative bacteria are MexA-MexB-OprM, MexE-MexF-OprN and MexC-MexD-OprJ in *Pasteurella aeruginosa*, and AcrA-AcrB-TolC and marR–efflux system in *E. coli* (Hooper, 2001a; Chen and Lo, 2003). Efflux pumps in Gram-positive bacteria contributing to fluoroquinolone resistance are NorA pumps (*Staphylococcus aureus*), LfrA efflux pumps (*Mycobacterium* sp.) and an unidentified energy-dependent efflux pump (*Enterococcus faecalis and E. faecium*) (Hooper, 2001a; Chen and Lo, 2003).

Another way to resistance is achieved by loss of porins, preventing enrofloxacin from entering the bacterial cell and reducing intracellular drug accumulation (Chen and Lo, 2003). This is achieved by decreased production of outer membrane proteins (Omps), alterations to the structure or composition of the Omps or the synthesis of novel Omps. Particularly OmpF and OmpC are involved (Chen and Lo, 2003). This mechanism also leads to decreased permeability to unrelated antibiotics (i.e. β-lactams, aminoglycosides, tetracyclines and chloramphenicol) (Brown and Reeves, 1997). However, this mechanism is not universal on

fluoroquinolone resistance as *Salmonella sp.* lacking OmpF porins did not show a decrease in fluoroquinolone accumulation (Chen and Lo, 2003).

Recently, plasmid-mediated horizontally transferable genes encoding quinolone resistance (*Qnr* genes) have been discovered (Robicsek *et al.*, 2006a). QnrA has been shown to bind DNA gyrase directly as well as topoisomerase IV. Hereby, QnrA minimizes opportunities for the quinolones to stabilise the lethal gyrase-DNA-quinolone cleavage complex (Tran *et al.*, 2005). According to several epidemiological surveys, QnrA was found in all populated continents (except South America) and in most clinically common *Enterobacteriaceae* (Robicsek *et al.*, 2006a).

Although these mechanisms do not allow a population to survive in the presence of a quinolone, as they only confer low-level resistance, they substantially enhance the number of resistant mutants that can be selected from the population. To define the proportion of clinical *Enterobacteriaceae* harbouring such low-level resistance and the effect of these genes on clinical outcome, further investigations are to be carried out (Robicsek *et al.*, 2006a).

1.1.5. Drug interactions and toxic effects of enrofloxacin in poultry and turkeys

To prevent a reduction in the antibacterial activity expected from enrofloxacin, and to optimize its clinical efficacy, some precautions must be observed when administering the drug to turkeys. Despite of analogue formulations, some enrofloxacin products lack bioequivalence which may result in lower maximum serum concentrations (Sumano *et al.*, 2001, 2006). Enrofloxacin is a photosensitive molecule. Exposure of medicated water to direct light must be prevented (Sumano *et al.*, 2004). Compounds containing divalent or trivalent cations, such as aluminium, calcium, iron, magnesium or zinc, administered concurrently with enrofloxacin, may reduce absorption of the fluoroquinolone (Walker, 2000b). Therefore, the use of galvanized water tanks, hard water as a vehicle, or food rich in magnesium or calcium should be avoided (Tanner, 2000; Sumano *et al.*, 2004).

Only few drug interactions with enrofloxacin are reported and most are of no veterinary significance. In chickens, enrofloxacin has been shown to inhibit liver microsomal mixed-function oxidases in broiler chickens, including aniline hydroxylase and aminopyrine *N*-demethylase (Shlosberg *et al.*, 1995) resulting in reduced elimination of drugs that depend on liver metabolism for excretion. Adverse interactions with ionophore antibiotics may therefore be anticipated. Cytochrome P450 enzyme activity was not significantly affected in chickens (Shlosberg *et al.*, 1995). In turkeys, reports concerning enrofloxacin depressing hepatic enzyme activity can not be found, but since the pharmacokinetics of enrofloxacin in turkeys

can be characterized as similar to those in chickens (Dimitrova *et al.*, 2006), the same measures should be taken.

Fluoroquinolones are relatively safe antimicrobials (Anonymous, 2003). Administered at therapeutic doses, toxic effects are mild and generally limited to gastrointestinal disturbances (Walker, 2000b). However, increased uptake of water medicated with enrofloxacin (e.g. because the temperatures in the stable are fluctuating) can cause cartilage lesions leading to arthropathies (Enrofloxacin package insert, Baytril, Bayer-US, Rev 11/00. Rec 6/10/02). Overdoses of enrofloxacin have been reported after administration of 626 ppm enrofloxacin in the drinking water to 1–day old turkey poults for 21 days. In the first 10 days, 11 out of 40 turkeys died. Surviving birds showed signs of listlessness and decreased body weight gain (Veterinary Healthcare Communications, 2001). For enrofloxacin no evidence of carcinogenicity was found in any study of laboratory animal models (Veterinary health care communications, 2001). The effect on reproduction in turkeys has not yet been established. In chickens no effects were noted measuring the reproductive parameters (egg production, egg weight, hatchability, chick viability and reproductive histology of treated birds and their progeny). In this study, male and female chickens were given enrofloxacin at a dose of 150 ppm in drinking water for 7 days, at different ages between 1-206 days (Veterinary Healthcare Communications, 2001). Because enrofloxacin is irritating to eyes and skin, safety precautions should be taken when administering the drug.

1.1.6. Pharmacokinetic parameters of enrofloxacin

The pharmacokinetic parameters of a drug are largely governed by its chemical nature and related physicochemical properties (Baggot, 2000). They include the route of administration, the rate of absorption, the rate of distribution, the volume of distribution, the protein binding capacity of the drug and the route and rate of elimination (Walker, 2000b).

Many reports can be found dealing with the pharmacokinetic data of enrofloxacin and species-specific differences have been observed (Haritova *et al.*, 2009). Because the experiments in this dissertation are performed in turkeys, mainly the pharmacokinetic properties of enrofloxacin in this bird will be discussed.

The pharmacokinetic values of enrofloxacin reported below were obtained after single (oral/intravenous) pulse administration (Haritova *et al.*, 2004; Dimitrova *et al.*, 2007) or after continuous oral dosing (Fraatz *et al.*, 2006) of turkeys at a dose of 10 mg/kg body weight/day. No significant differences were noticed between sexes (Dimitrova *et al.*, 2006).

After oral administration, enrofloxacin is well absorbed. The oral bioavailability of the drug proved to be 69.20% (Dimitrova *et al.*, 2007). The mean absorption time was 2 hours and 45 minutes and maximum serum concentrations (i.e. 1.23 µg/ml) were reached approximately 6 hours after treatment (Dimitrova *et al.*, 2007). From 10 minutes until the 24th hour inclusive, serum levels remained above 0.170 µg/ml. After intravenous injection serum levels were constantly higher than 0.20 µg/ml (Dimitrova *et al.*, 2007). After continuous drinking water medication (10 mg/kg for 3 continuous days), mean serum concentrations at day three of treatment ranged between 0.32-0.45 µg/ml (Fraatz *et al.*, 2006). Following absorption, enrofloxacin is well distributed in different tissues. Volume distributions of approximately 4 l/kg were observed (Haritova *et al.*, 2004; Dimitrova *et al.*, 2007). At the third day of continuous enrofloxacin administration, mean concentrations in liver and lung ranged between 3.49-3.58 and 0.95-1.09 µg/ml, two to three times higher than the mean concentrations found in serum (Fraatz *et al.*, 2006), indicating very good drug penetration into the deep respiratory tract. Enrofloxacin concentrations are higher in infected tissues compared to healthy tissues, probably because fluoroquinolones rapidly accumulate in leukocytes. In addition, because they are distributed into the cytosol, intracellular pathogens can be reached (Papich and Riviere, 2001). The elimination half-life ($t_{1/2}$) of enrofloxacin is long, making the drug ideal for q12-q24-hour dosing regimens. After single oral administration at 10 mg/kg body weight $t_{1/2}$ was 6.92 +/-0.97 h. The mean residence time (MRT) was 11.91 +/- 1.87 h. After intravenous injection $t_{1/2}$ was 6.64 h (+/- 0.90 h) and MRT 8.96 h (+/- 1.18 h) (Dimitrova *et al.*, 2007). Enrofloxacin is partially metabolized in the liver, yielding the metabolites ciprofloxacin, dioxociprofloxacin, oxociprofloxacin, *N*-formyl ciprofloxacin, enrofloxacin amide, desethylene enrofloxacin, oxoenrofloxacin and hydroxyl oxoenrofloxacin (Dimitrova *et al.*, 2007). The major metabolite is ciprofloxacin, the only metabolite with antimicrobial activity (Anonymous, 2003). In turkeys, a fast but low-level biotransformation of ciprofloxacin can be observed with serum concentrations being $\leq$ 7.68% of the parent substance (Fraatz *et al.*, 2006; Dimitrova *et al.*, 2007). Also in body tissues, ciprofloxacin levels were low ($\leq$ 5% of the total antimicrobial concentration) except in the liver where levels exceeded 40% (Fraatz *et al.*, 2006). However, because of the usually lower MIC of ciprofloxacin for pathogens, Dimitrova *et al.* (2007) evaluate the combined action of enrofloxacin and ciprofloxacin as important. Besides the bile, enrofloxacin is mainly excreted in the urine (Dimitrova *et al.*, 2007). Reported body clearance (Clb) values are 0.47 l/kg/h (Haritova *et al.*, 2004) and 0.41 l/kg/h (Dimitrova *et al.*, 2007).

The pharmacokinetics of enrofloxacin can be characterized as similar to those in chickens (Dimitrova, 2007). Half-life and mean maximum serum concentrations are almost the same as in chickens (Bugyei *et al.*, 1999; Garcia Ovando *et al.*, 1999; Sumano *et al.*, 2001). In contrast with chickens, the process of absorption after oral administration seems delayed and also the biological half-life and mean residence time are longer (Anadon *et al.*, 1995; Abd- El-Aziz *et al*., 1997; Bugyei *et al.*, 1999; Knoll *et al.*, 1999; Sumano *et al*., 2001). A hypothetical cause for these differences could be the experimental conditions, the species and breed peculiarities, and the difference in the body weight of the treated birds (Dimitrova *et al.*, 2007).

1.1.7. Pharmacodynamic parameters of enrofloxacin

Antimicrobial pharmacodynamics describe the impact of an antimicrobial agent on a target pathogen and are based on the drug's pharmacokinetics and microbiological activity toward that pathogen, together with the pathogen's susceptibility (Rybak, 2006). Pharmacodynamic properties include drug concentration over time in the tissue and other body fluids, drug concentration over time at the site of infection, and antimicrobial effect at the site of infection. Pharmacodynamic antimicrobial measures and effects at the site of infection include minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), concentration-dependent killing effect, post-antibiotic effect (PAE), sub-MIC post-antibiotic effect (SM-PAE), and post-antibiotic leukocyte enhancement effect (PALE) (Walker, 2000b).

The minimal inhibitory concentration or MIC of an antimicrobial is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a standardised inoculum of approximately 10^5 cfu/ml micro-organisms after overnight incubation (Andrews, 2001). The MICs that inhibit 50% and 90% of a collection of field isolates are defined as MIC_{50} and MIC_{90}, respectively. MICs have a pivotal role as prime pharmacodynamic predictor factor in designing dosing regimens (Frimodt-Moller, 2002). Although low MICs generally indicate greater *in vitro* potency, these values must be interpreted in relation to achievable serum and tissue concentrations of the drug (Walker, 2000a). Bacteria with MICs $\leq$ 0.25 µg/ml are regarded susceptible to enrofloxacin, bacteria with MICs between 0.5-1 µg/ml intermediate susceptible and bacteria with MICs $\geq$ 2 µg/ml considered resistant to enrofloxacin (CSLI guidelines, M31-A3, 2008).

Enrofloxacin exhibits bactericidal action. The minimal bactericidal concentration (MBC) is the lowest concentration of an antimicrobial drug that causes at least a 3 $\log_{10}$ reduction in the

number of surviving bacterial cells compared to the initial pre-incubation inoculum after 18-24 hours of incubation (Prescott and Walker, 2000). For enrofloxacin, the bactericidal concentrations are equal to or barely twice as high as the MIC (Pirro *et al.*, 1997).

Enrofloxacin exerts a concentration-dependent killing. Whenever the concentration of the fluoroquinolone increases above the MIC of the pathogen, the number of viable organisms decreases dramatically, because of increased killing (Prescott and Walker, 2000). As the ratio of enrofloxacin concentration to MIC increases from 1:1 to the bactericidal concentration, a bacterial reduction of more than 3 $\log_{10}$ cfu/ml (i.e. 99.9% of the bacteria) is achieved within 6 hours of exposure (Maxwell and Critchlow, 1998). Unfortunately, fluoroquinolones also exhibit a biphasic dose response curve. They are less active at concentrations below or much higher than the MIC (Brown, 1996). As mentioned before, the decrease in antimicrobial activity at high drug concentrations is thought to be caused by inhibition of protein or RNA synthesis, resulting in bacteriostasis (Smith and Zeiler, 1998).

The post-antibiotic effect (PAE) of enrofloxacin refers to the persistent suppression of bacterial growth following initial exposure and subsequent removal of the drug. The length of the PAE depends on the microorganism, the duration of exposure and the concentration of the drug to which the bacteria are exposed (Prescott and Walker, 2000). At concentrations of 10 times the MIC prior to drug withdrawal, the PAE of enrofloxacin for *Pasteurella multocida* was 3.2 h (Fera *et al.*, 2002). At concentrations of 8 times the MIC prior to drug withdrawal, the PAE of enrofloxacin for *E. coli* was 1.9 h (Wang *et al.*, 2003).

When there is a slow removal of the drug from the site of infection, a sub-inhibitory drug concentration may continue to inhibit DNA and protein synthesis (Prescott and Walker, 2000). This is called the sub-MIC post antibiotic effect and can be measured by exposing the bacterium to a concentration corresponding to half the MIC, following primary drug exposure and drug removal (Prescott and Walker, 2000). This effect is important for pulsed-dose medication, as enrofloxacin concentrations decline for some time below level of bacteriostatic or bactericidal activity between successive medication days (Baggot, 2000). Because of its variable duration, generally 1-6 hours, PAE is not taken into account when calculating dosage regimens (Baggot, 2000).

Finally, for enrofloxacin a post-antibiotic leukocyte enhancement effect (PALE) is reported. PALE describes a bacterium's increased susceptibility to phagocytosis and intracellular killing following drug exposure (Prescott and Walker, 2000) and can be ascribed to the immunomodulatory effects of enrofloxacin.

1.1.8. PK/PD parameters defining dosing strategies

To predict the efficacy of an antimicrobial compound, specific PK/PD parameters are commonly used. For fluoroquinolones, which are concentration-dependent, these parameters are the ratio of maximum serum concentration to the minimum inhibitory concentration C_{max}/MIC and the ratio of the area under the curve of the plasma concentration over time (AUC) to the MIC (AUC/MIC) (Frimodt-Moller, 2002; Ryback, 2006). The higher these ratios are, the better the cure rate (reduction of clinical symptoms, clearance of the pathogen from the site of infection) and the lower the chance that less drug-sensitive or drug-resistant microorganisms will emerge (Frimodt-Moller, 2002). Athough a strong correlation exists between both ratios (correlation coefficient of 0.92) (Forrest *et al.*, 1993), the AUC/MIC ratio correlates best with clinical outcome and the C_{max}/MIC ratio is the more appropriate measure for assessing the potential for emergence of less susceptible or resistant microbial subpopulations (Ambrose, 2003).

Many studies, primarily performed in Gram-negative bacteria, have concluded that for clinical and microbiological success and to limit the development of bacterial resistance AUC/MIC ratios of >100-125 or C_{max}/MIC ratios of >10 are required (Blaser *et al.*, 1987; Madaras-Kelly *et al.*, 1996, Frimodt-Moller, 2002). In turkeys, the pharmacodynamic predictors of enrofloxacin, orally given at 10 mg/kg body weight, for clinically significant microorganisms whose MIC varied from 0.008 µg/ml to 0.125 µg/ml were determined by Dimitrova *et al.* (2007). C_{max}/MIC ranged from 161.23 +/- 5.9 h to 12.90 +/-0.5 h and $AUC_{0\rightarrow24}$/MIC rated from 2153.44 +/- 66.6 h to 137.82 +/- 4.27 h. Similar results were found by Fraatz *et al.* (2006) whereas mean enrofloxacin concentrations in the lung after continuous drinking medication at 10 mg/kg body weight exceeded MIC90 values for *E. coli* (0.06 µg/ml) by approx four $\log_2$, for *P. multocida* (0.16 µg/ml) by six steps, for *O. rhinotracheale* (0.25 µg/ml) by two steps, for *M. gallisepticum* (0.1 µg/ml) by three steps and *M. synoviae* (0.5 µg/ml) by one step. These high values should provide a maximal clinical and microbiological effect, and a good condition to minimize the risk of appearance of antimicrobial resistance in this bird species.

Certain limitations are involved with the application of the pharmacodynamic predictors in designing dosing strategies. First, there is a considerable variation in pharmacodynamic measures and outcome parameters appear to be quinolone and pathogen specific. Secondly, unsolved questions regarding optimal pharmacodynamic outcome predictors for Gram-positive bacteria, anaerobes and atypical respiratory pathogens remain (Wright *et al.*, 2000).

In addition, Zhao and Drlica (2008) argue that dosing strategies based on the previous predictors fail to consider resistant mutant subpopulations as only bulk population susceptibility, measured by MIC, is being addressed. Therefore, another strategic approach is to use the mutant selection window hypothesis (Drlica, 2003). The mutant selection window is an antimicrobial concentration range extending from the minimal concentration required to block the growth of wild-type bacteria (MIC_{99}) up to that required to inhibit the growth of the least susceptible, single-step mutant. The upper boundary is called the mutant prevention concentration (MPC). Consideration of the mutant selection window leads to the suggestion that antimicrobial concentrations between MIC and MPC enrich mutant subpopulations selectively. If antimicrobial concentrations are kept above mutant prevention concentration throughout therapy, mutant subpopulation (single-step mutants) amplification will be inhibited (Zhao and Drlica, 2008). For fluoroquinolones the MPC corresponds to AUC_{24}/MIC > 200 (Drlica, 2003). However, when an antimicrobial agent like enrofloxacin for example has two intracellular targets (DNA gyrase and topoisomerase IV), a bacterial cell would have to acquire concurrent mutations for growth. As mutations occur at a rate of 10^{-14} and bacterial populations in infected individuals only reach 10^{10} cells, resistant mutants would be rarely recovered as the mutant prevention window would be closed (MIC = MPC) (Ng *et al.*,1996, Pan *et al.*, 1996). Whether exceeding the MPC, which is an *in vitro* concept, is sufficient to restrict the development of resistance *in vivo* requires clinical testing.

Another dosing strategy aiming at slowing down the emergence of mutational resistance is dose escalation modelling. It involves mathematical modelling of data obtained with escalating doses to identify the susceptible population-based drug exposure (AUC_{24}/MIC) that blocks amplification of resistant mutant subpopulations and kills most susceptible cells. As in the first strategy, problems arise from using bulk population susceptibility rather than mutant subpopulations susceptibility (Jumbe *et al*., 2003). Instead, using the MPC-based threshold AUC_{24}/MPC as a target parameter for blocking resistant subpopulation proliferation would improve the model (Zhao and Drlica, 2008). This index for susceptible cells should apply to resistant subpopulations if MPC is substituted for MIC, as MPC correlates with the MIC of the least-susceptible single (next)-step mutant. However, more *in vivo* studies must be carried out to confirm these expectations.

1.2. MULTICAUSAL RESPIRATORY DISEASE IN TURKEYS

Respiratory disease in turkeys is complex and involves multiple etiologies. Unfavourable environmental and husbandry factors such as high atmospheric ammonia levels, high dust levels and changes in temperature predispose for infectious respiratory agents (Kleven, 2008). In addition, immunosuppressive agents like hemorrhagic enteritis virus or digestive disorders may weaken the immune system rendering the turkeys more susceptible to respiratory infections (Kleven, 2008). These respiratory infections can be implicated by several pathogenic agents (Kleven, 2008). Avian metapneumovirus, Newcastle disease, Paramyxovirus 1, 2 and 3 and avian influenza are relevant associated viral infections. Also severe or prolonged vaccination reactions following the use of live ND or infectious bronchitis vaccines may result in the development of respiratory disease. Bacterial species commonly involved are *Mycoplasma* sp., *E. coli*, *O. rhinotracheale*, *P. multocida*, *Riemerella anatipestifer*, *Bordetella avium* and *Chlamydophila psittaci*. Finally moulds like *Aspergillus fumigatus* can also be implicated in turkey respiratory disease. However, certain infections are more encountered in the commercial turkey production than others. The viral agent currently most relevant for commercial turkey production and acting as a primarily triggering agent is avian metapneumovirus. *E. coli* and *O. rhinotracheale* are the two most frequently encountered micro-organisms causing secondary bacterial infections and severely aggravate the turkey respiratory disease and worsen its economic impact. In the next paragraphs these two important bacterial species are more thoroughly reviewed.

1.2.1. *Ornithobacterium rhinotracheale* infections in turkeys

1.2.1.1. A short history and general characteristics of *O. rhinotracheale* infections

In 1991 a new respiratory disease characterized by a foamy white, yoghurt-like exudate in the air sacs in South African broiler chickens was reported (van Beek *et al.*, 1994). Bacterial examination revealed a "*Pasteurella*-like" bacterium, that could not be classified as any of the known bacterial species. Thereupon, organisms with the same feature were isolated out of poultry, including turkeys, in the Netherlands and Germany (Hafez *et al.*, 1993; Hinz *et al.*, 1994; Van Beek *et al.*, 1994). The new organism was designated several names. It was referred to as *Pasteurella*-like bacterium (Hafez *et al.*, 1993) and *Kingella*-like bacterium (Van Beek *et al.*, 1994). Charlton *et al*. (1993), the first ones to characterize the bacterium, called it a pleomorphic Gram-negative rod and Bisgaard (van Beek *et al.*, 1994) stated that it

could be classified within a group of bacteria designated TAXON 28. In 1994 Vandamme *et al*. (1994) proposed its custom name -*Ornithobacterium rhinotracheale*- after identifying its phylogenetic position and various genotypic, chemotaxonomic, and classical phenotypic characteristics. *O. rhinotracheale* is a member of the rRNA superfamily V within the *Cythophaga-Flavobacterium-Bacteroides* phylum and closely related to *Riemerella anatipestifer* and *Coenonia anatine*. Afterwards, investigations of culture collections (Hinz and Hafez, 1997) revealed that *O. rhinotracheale* was already isolated from turkeys and rooks suffering from respiratory disease in Germany, England and California in the early eighties, years before the first observation in South Africa.

Since its recognition in 1994, numerous isolations of *O. rhinotracheale* have been reported from many countries worldwide and out of a whole range of avian species: partridge, pheasant, pigeon, rook, quail, duck, chuckar partridge, ostrich, goose, guinea fowl, chicken and turkey (Charlton *et al.*, 1993; Vandamme *et al.*, 1994; Devriese *et al.*, 1995; Buys, 1996; Abdul-Aziz *et al.*, 1999; Joubert *et al.*, 1999; Sakai *et al.*, 2000). Thus, it can be assumed that the organism is spread all over the world.

After incubation, oxidase-positive, catalase-negative, nonhemolytic, circular, grey to grey-white, 1-2 mm convex colonies with an entire edge pin point and a butyric acid odor will appear (Chin *et al.*, 2008). After Gram staining, a characteristic pleomorphic, rod-shaped, nonsporulating, Gram-negative bacterium measuring 0.2-0.9 µm in width and 0.6-5 µm in length is noticed (Chin *et al.*, 2008). The most characteristic biochemical properties of *O. rhinotracheale* are: positive for oxidase, urease, β-galactosidase, arginine dehydrolase, alkaline phosphatase, esterase lipase, leucine arylamidase, cystine arylamidase, acid phosphatase, phosphohydrolase, α-glucosidase, N-acetyl-β-glucosaminidase, and acid production from glucose, fructose, lactose and galactose. The bacterium is negative for nitrate reduction, catalase, growth on McConkey agar, motility, lysine decarboxylase, indole production, gelatinase, esterase, lipase, chymotrypsin, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, and acid production from maltose, sucrose, fructose and ribose (van Empel and Hafez, 1999).

Currently, 18 serotypes (A through R) of *O. rhinotracheale* have been determined. In turkeys, 61% of the isolates belong to serotype A. Since serotype C could only be isolated from turkeys in South Africa and the United States, there appears to be a correlation between the geographic origin of the isolates and their serotype. There is no indication of host specificity of the serotypes (Chin *et al.*, 2008).

1.2.1.2. Pathogenesis and epidemiology of *O. rhinotracheale* infections

Respiratory disease has been and continues to be a significant problem for the turkey industry. Like in all poultry species, infections of the respiratory tract in turkeys can rarely be attributed to a single agent. Under commercial conditions complicated infections involving multiple etiologies with viruses, mycoplasmas, other bacteria, immunosuppressive agents and unfavourable environmental conditions are the rule rather than the exception. Besides, the use of live viral vaccines against Newcastle Disease or Infectious Bronchitis play a major role in the development of respiratory disease (Kleven, 2008).

Taking in consideration the many reports of field cases of *O. rhinotracheale* infections, it is clear that the organism, twenty years after its discovery as the cause of a new respiratory disease in poultry (van Beek *et al.*, 1994), plays a significant role in the respiratory disease complex. As mentioned above case reports of uncomplicated *O. rhinotracheale* infections in turkeys are scarce and usually concomitant infections with other respiratory pathogens such as Newcastle disease virus, avian metapneumovirus, *Mycoplasma synoviae, Mycoplasma gallisepticum*, *Escherichia coli*, *Bordetella avium* and *Chlamydophila psittaci* are seen in the field. Several researchers (DeRosa *et al.*, 1997; Chin and Droual, 1997; van Empel and Hafez, 1999, Zorman-Rojs, 2000, Van Loock *et al.*, 2005; Marien, 2007) have succeeded in demonstrating the synergistic effect between *O. rhinotracheale* and these other respiratory pathogens frequently encountered in the field showing aggravating clinical symptoms when turkeys inoculated with *O. rhinotracheale* were challenged with concomitant infections.

The precise role of *O. rhinotracheale* as either a primary or secondary pathogen is not clear yet. Most experimental studies have concluded that, when *O. rhinotracheale* is experimentally inoculated as single agent, the organism causes minimal pathological lesions (Back *et al.*, 1997; De Rosa *et al.*, 1997; Droual and Chin, 1997; Jirjiss *et al.*, 2004; Marien *et al.*, 2005). Upon aerosol, oculonasal or intra-tracheal inoculation with *O. rhinotracheale* of specified pathogen free (SPF) birds, pneumonia or airsacculitis were never induced with *O. rhinotracheale* alone. Only after intravenous challenge meningitis, osteitis and a mortality rate of 20% could be induced in SPF chickens (Goovaerts *et al.*, 1999). Airsacculitis, however, a lesion often observed in the field, was not reported in the experiment. In contrast with the situation in SPF birds, some research groups did observe pathological lesions similar to those seen in field cases after experimental infection of commercial birds with *O. rhinotracheale*. Ryll *et al.* (1996) and Sprenger *et al*. (1998), for example, observed airsacculitis, pneumonia and increased mortality after aerosol, intra-tracheal, intravenous and/or intra-thoracic *O. rhinotracheale* inoculation. Also Van Empel *et al.* (1996) could evoke airsacculitis and

growth retardation after aerosol and intra-air sacs challenge. However, using commercial birds in experimental studies implies that their microbiological and immunological status are uncertain, which might have contributed to the development of lesions. Also the route of infection used such as intrasaccular inoculation may bypass the bird's innate defence mechanisms situated at the upper respiratory tract.

Although the question whether *O. rhinotracheale* is or is not a primary respiratory pathogen is still under debate, several studies (mostly conducted in chickens) have reported differences in pathogenicity between isolates of *O. rhinotracheale*. After inoculation into the air sacs of broilers, three South African isolates showed significant differences in the production of airsacculitis and arthritis (Travers *et al.*, 1996). In another study in chickens, an American *O. rhinotracheale* isolate proved to be less pathogenic compared to Dutch and South African isolates (Van Veen *et al.*, 2000). In addition, Hafez and Popp (2003) classified several *O. rhinotracheale* isolates as pathogenic, moderately pathogenic or highly pathogenic on the basis of embryo lethality tests in chickens and turkeys. The existence of pathogenicity difference between *O. rhinotracheale* isolates could be a plausible explanation why some *O. rhinotracheale* strains appear to produce respiratory disease on their own and other isolates do not.

Knowledge of the mechanisms of virulence of *O. rhinotracheale* is still very limited. Currently no special structures or properties such as pili, fimbriae or specific toxic activities have been reported (Chin *et al.*, 2008). A study investigating the role of outer membrane proteins (Omp) in the interaction of *O. rhinotracheale* with tracheal epithelium showed that antibodies against a 53 kDa Omp resulted in a significant inhibition of bacterial adherence to tracheal epithelium of up to 78% (Nofouzi *et al*., 2007). Another study, investigating the iron acquisition mechanism of *O. rhinotracheale*, observed that several field strains of *O. rhinotracheale* were resistant to the iron chelator 2,2'-dipyridyl, suggesting that this attribute may be related to disease-producing potential of these strains (Tabatabai *et al*., 2008). It has been reported that one strain of *O. rhinotracheale*, belonging to the rare serotype K, carries a cryptic plasmid pOR1 (Back *et al*., 1997; Jansen *et al*., 2004). The plasmid was probably introduced on a single recent occasion as it has not spread in the population. However, based on this cryptic plasmid pOR1, a construct plasmid shuttle vector pOREC1 has recently been constructed (Jansen *et al*., 2004). This transformation of *O. rhinotracheale* could pave the way to genetic manipulation of this bacterium and could aid in a better understanding of the virulence producing potential of *O. rhinotracheale* strains.

Transmission of *O. rhinotracheale* occurs horizontally by direct contact or indirectly through aerosols or drinking water. Since the bacterium has been isolated from the sexual apparatus (ovaries and oviduct), hatching eggs, infertile eggs, dead embryos and dead-in-shell turkeys, *O. rhinotracheale* also seems to spread vertically (Chin *et al.*, 2008), which was confirmed by experimental studies performed by van Veen *et al.* (2004). Outbreaks of *O. rhinotracheale* infections occur more often during the winter months (Chin *et al.*, 2008). This is explained by the longer survival period of the organism at lower temperatures (6 days at 22°C, 40 days at 4°C) (Lopes *et al.*, 2002c).

1.2.1.3. Clinical symptoms observed during *O. rhinotracheale* outbreaks in turkeys

O. rhinotracheale can cause an acute, highly contagious disease in turkeys. The severity of clinical signs, the duration of the disease and the mortality rate of *O. rhinotracheale* outbreaks are, however, extremely variable. They can be influenced by concurrent diseases, secondary infections, and possible also due to a pathogenicity difference between *O. rhinotracheale* strains. Viruses affecting the respiratory tract like avian metapneumovirus, Newcastle disease virus or Infectious bronchitis virus are regarded as important triggering agents. In addition, environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, high ammonia levels, can seriously aggravate clinical disease (Van Empel and Hafez, 1999; Chin *et al.* 2008).

Generally, the clinical disease observed in turkeys is more severe than in broilers (Chin *et al.*, 2008). Initial symptoms are coughing, sneezing and nasal discharge, followed, in some cases, by severe respiratory disease, prostration and sinusitis. These symptoms are accompanied with a reduction of feed consumption and water intake (Chin et al, 2008). In turkey breeder flocks, a decrease in egg production and an increase in the number of unsuitable hatching eggs can be observed (De Rosa et al, 1996; Van Empel and Hafez, 1999). In addition, *O. rhinotracheale* has been reported to cause nervous signs, paralysis (motor disturbances, recumbency, abnormally carriage of the head), arthritis, meningitis, osteitis and osteomyelitis (Van Empel and Hafez, 1999; Szalay *et al.*, 2002). Turkeys are mostly affected between 2 and 8 weeks of age and also when older than 14 weeks (Chin *et al.*, 2008). Mortality rates of up to 50% are reported, but normally mortality ranges between 1-15% (Chin *et al.*, 2008). Clinical disease is more severe in heavier birds. The higher mortality in toms than in hens has therefore been associated with their higher weight (DeRosa *et al.*, 1996).

1.2.1.4. Macroscopic and microscopic lesions associated with *O. rhinotracheale* infections in turkeys

Gross lesions associated with *O. rhinotracheale* infections in turkeys are rhinitis, edema and unilateral or bilateral consolidation of the lungs and fibrinous exudate in the air sacs and on the pleura. In addition, mild tracheitis, peritonitis and pericarditis occasionally occur (van Empel and Hafez, 1999; Chin *et al.* 2008). Besides, yoghurt-like exudate in the air sacs, a feature usually seen in chickens, is sometimes observed (personal observation). In some cases, swelling of the liver and spleen, as well as degeneration of heart muscles, have been noticed (van Empel and Hafez, 1999). In older birds infection of the joints and vertebrae can be seen (Chin *et al.*, 2008) and Szalay *et al.* (2002) report fibrinopurulent inflammation of the cranial bones and meningitis.

The following microscopic lesions have been associated with *O. rhinotracheale* infections in turkeys in field cases (De Rosa *et al.*, 1996; Chin *et al.*, 2008): congested lungs, large collections of fibrin admixed with macrophages and heterophils lying free within the lumen of air capillaries and parabronchi through the parenchyma, pronounced and diffuse interstitial infiltrates of macrophages with smaller numbers of heterophils, coalescing foci of necrosis usually filled with dense aggregates of necrotic heterophilic infiltrate or exudate and often centered within the lumen of parabronchi with extension of the necrosis into the adjacent parenchyma. Within these necrotic foci, small clusters of bacteria can be scattered. Also severely thickened and edematous pleura and air sacs with interstitial fibrin deposits, diffuse heterophilic infiltrate, scattered small foci of necrotic heterophilic infiltrate, and fibrosis have been seen (De Rosa *et al.*, 1996; Chin *et al.*, 2008). Experimental infections with *O. rhinotracheale* in turkeys (Marien *et al.*, 2005) also describe an inflammatory reaction in the mucosa, loss of cilia and degeneration of mucous glands of the turbinates and trachea. These experiments, however, showed that *O. rhinotracheale* alone was only able to cause minor histological abnormalities. Viral triggering (avian metapneumovirus) resulted in histological lesions more comparable to those described in the field cases of De Rosa *et al.* (1996).

1.2.1.5. Diagnosis of *O. rhinotracheale* infections in turkeys

Lesions associated with *O. rhinotracheale* infections in turkeys are similar to those caused by numerous other bacteria affecting the respiratory tract, such as *Escherichia coli, Pasteurella multocida, Riemerella anatipestifer* and *Chlamydophila psittaci*. Therefore, clinical signs and necropsy findings are of little value for diagnosis (Van Empel and Hafez, 1999; Chin *et al.*, 2008) and bacterial culture is best carried out.

Isolation of *O. rhinotracheale* occurs preferably from the trachea, tracheal swabs, lungs and air sacs. Also the nasal cavity and the infraorbital sinuses can be used for isolation. However, because of overgrowth of other bacteria, *O. rhinotracheale* can be easily masked (Van Empel and Hafez, 1999; Chin *et al.*, 2008). Under field conditions, *O. rhinotracheale* has also been isolated out of the pericardium and peritoneum (De Rosa *et al.*, 1996) but until now never from the heart, spleen or liver (van Empel and Hafez, 1999; Chin et al., 2008). After experimental *O. rhinotracheale* infection, however, positive cultures from these organs have been obtained as well as from the joints, brains, ovary and oviduct and kidney (van Beek *et al*., 1994; Back *et al*., 1998b; Sprenger *et al.*, 1998; Marien *et al.*, 2005).

The conditions for optimal growth are incubation on Columbia agar with 5% sheep blood (sheep blood agar) for at least 48 hours in a CO_2-enriched atmosphere (5-10% CO_2). To suppress overgrowth by fast-growing bacteria, gentamicin and polymyxin can be added to the sheep blood agar, both at 5 µg/ml. However, sheep blood agar without these additives should always be included, since only 90% of *O. rhinotracheale* strains are resistant to these antimicrobials (van Empel and Hafez, 1999). *O. rhinotracheale* grows readily on tryptose soy agar, on chocolate agar as well as in peptone water and pasteurella broth aerobically and anaerobically (Hafez, 1998; Chin *et al.*, 2008).

Based on its biochemical properties, identification of *O. rhinotracheale* can be done using the API-20NE system (BioMèrieux, France) after which 99.5% of the *O. rhinotracheale* strains result in a code 0-2-2-0-0-0-4 or 0-0-2-0-0-0-4 (Hafez, 1998). The API-ZYM systems (BioMèrieux, France) result consistently in negative reactions for lipase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase and high identification scores (4-7-2-2-6-4, 4-7-6-2-6-4, 6-7-6-2-6-4, 6-7-2-2-6-4) were also seen using the RapID NF Plus system (Innovative Diagnostics, USA) (Post *et al*., 1999). In addition, identification of the fatty acid profile (Charlton *et al.*, 1993), a rapid slide agglutination test (Back *et al.*, 1998) and an agar gel precipitation (van Empel *et al.*, 1997) can be used to identify *O. rhinotracheale* isolates.

Immunohistochemical staining, immunofluorescence staining and PCR assays are also useful for identification purposes (van Empel and Hafez, 1999; Chin *et al.*, 2008). 16sRNA gene sequencing and also repetitive-PCR fingerprinting techniques using the primers ERIC (enterobacterial repetitive intergenic consensus) 1R and M13 have been used as well (van Empel and Hafez, 1999). Both ERIC 1R and M13 revealed different rep-PCR patterns, but the M13 fingerprinting technique was found to be more discriminating in differentiating *O. rhinotracheale* isolates to the serotype level (Amonsin *et al.*, 1997; Thachil *et al.*, 2007).

Finally, serology is efficient as an aid in the diagnosis of *O. rhinotracheale* and is also useful for flock monitoring. Testing for antibodies has been described by serum plate agglutination (Back *et al.*, 1998a), agar gel precipitation, immunoperoxidase test (van Empel *et al.*, 1997), DOT-immunobinding assay (Erganis et al, 2002) and enzyme-linked immunosorbent assays (also commercial ELISA kits are available (Biocheck, Gouda)) (Hafez, 2002).

1.2.1.6. Treatment of *O. rhinotracheale* infections in turkeys and preventive measures

Several antimicrobial sensitivity studies have demonstrated that acquired resistance against antimicrobials commonly used in poultry such as amoxicillin, ampicillin, doxycycline, enrofloxacin, flumequine, gentamicin, lincomycin, trimethoprim-sulfonamide, tetracycline and tylosin is very high (Devriese *et al*., 1995; 2001; Dudouyt *et al*., 1995; van Veen *et al.*, 2001; Soriano *et al*., 2003). As the susceptibility of *O. rhinotracheale* to antimicrobials is highly inconsistent and seems to depend on the geographical location of the strain (Chin *et al.*, 2008), antimicrobial treatment of *O. rhinotracheale* infections in turkeys and poultry should always go with vigilant sensitivity testing.

Several studies on vaccination of turkeys against *O. rhinotracheale* infection have been reported. Vaccinations based on inactivated whole-cell formulations (bacterins in oil adjuvant) have shown to induce protective immunity against homologous *O. rhinotracheale* infections in turkeys (van Empel and Hafez, 1999; Sprenger *et al.*, 2000, Van Veen *et al.*, 2004). Vaccination of turkey breeders with an injectable bacterin reduced vertical transmission but vaccination of the progeny is needed to resist challenge at 6 weeks of age (Van Veen *et al.*, 2004). Unfortunately, most of these bacterins in oil adjuvans do not induce cross-protection against other serovars (van Empel and Hafez, 1999). However, because of the high variety of serotypes in turkeys (only 61% of the *O. rhinotracheale* isolates in turkeys belongs to serotype A compared to 97% in chickens), protection against multiple serotypes is necessary. Shuijffel *et al.* (2005; 2006) succeeded in developing a four component subunit vaccine, based on recombinant cross-protecting antigens, that protects against challenge with a heterologous *O. rhinotracheale* serotype. A disadvantage of oil adjuvant vaccines is that they can cause local reactions and sometimes have negative effects on the performance of the birds (Chin *et al.*, 2008). Also, the way of administering these inactivated vaccines (subcutaneous injection) is costly and impractical in commercial flocks. Vaccination with attenuated *O. rhinotracheale* strains could avoid these negative side effects. Sprenger *et al.* (2000) vaccinated turkeys intranasally with a live *O. rhinotracheale* vaccine resulting in the protection of turkeys against pathologic changes after an intratracheal challenge with a

virulent *O. rhinotracheale*. Lopes *et al.* (2002a, 2002b) developed a temperature sensitive mutant of *O. rhinotracheale* and used it as a live vaccine administered in the drinking water to turkeys minimizing pathological changes after *O. rhinotracheale* infection. Roepke (2001) administered an autogenous live vaccine orally protecting turkeys against *O. rhinotracheale* infection even when the turkeys were simultaneously vaccinated with a live paramyxovirus 1 vaccine. This finding is remarkable, since in practice all investigated *O. rhinotracheale* strains have been pathogenic after viral priming (van Empel and Hafez, 1999) making practical use so far impossible.

1.2.2. Avian pathogenic *Escherichia coli* infections in turkeys

E. coli is a Gram negative, non-sporulating, rod-shaped bacterium of the family *Enterobacteriaceae* present in the normal microbiota of the avian intestinal tract. A certain number of these strains, however, designated as avian pathogenic *E. coli* (APEC), possess particular virulence attributes which enables them to cause disease responsible for severe economic losses to all poultry producers worldwide (Dho-moulin and Fairbrother, 1999; Vandekerchove, 2004; Barnes *et al.*, 2008; Dziva and Stevens, 2008).

Colibacillosis refers to any localized or systemic infection caused primarily or secondarily by APEC, and may include colisepticemia, air sac disease or chronic respiratory disease (CRD), swollen-head syndrome, osteomyelitis/synovitis or turkey osteomyelitis complex, coligranuloma aka Hjarre's disease, venereal colibacillosis, coliform cellulitis, peritonitis, salpingitis, orchitis, panophtalmitis, omphalitis/yolk sac infection and enteritis (Barnes *et al.*, 2008). Since already several thorough reviews on APEC and colibacillosis in poultry exist (Barnes *et al.*, 1994, 2008; Gross, 1994; Dho-Moulin and Fairbrother, 1999; Vandemaele *et al.*, 2002, Awers *et al.*, 2003; La Ragione *et al.*, 2004; Vandekerchove, 2004; Rodriguez-Siek *et al.*, 2005; Dziva and Stevens, 2008) the following paragraphs will focus on respiratory colibacillosis in turkeys.

1.2.2.1. Respiratory colibacillosis

Respiratory colibacillosis is the most common feature of colibacillosis in chickens and turkeys. Starting as a respiratory tract infection, it may evolve into bacteraemia and a generalized infection which manifests as a polyserositis (Barnes *et al.*, 2008; Dho-Moulin and Fairbrother, 1999). When the respiratory mucosa is damaged, APEC strains easily succeed in entering the blood stream (Barnes *et al.*, 2008). Excessive ammonia levels from poor

ventilation or faecal degradation in wet litter and fluctuations between low and high temperatures are non infectious agents that can primarily affect the respiratory tract. In addition, infectious agents such as avian metapneumovirus and *Mycoplasma* sp., but also live vaccine strains of Infectious bronchitis virus or Newcastle disease virus (Ficken *et al.*, 1987; Kleven *et al.*, 2008) can typically predispose the respiratory tract in turkeys. An increased susceptibility of turkeys to colisepticemia is also promoted by other bacterial respiratory pathogens like *O. rhinotracheale* and *Chlamydophila psittaci* (Barnes *et al.* 2008; Kleven *et al.*, 2008; Marien, 2008).

Lesions are prominent in the trachea, lungs, air sacs. Infected airsacs are thickened and often have caseous exudate in the respiratory surface. Common in turkeys are pneumonia and pleuropneumonia. Lesions in the pericardial sac and peritoneal cavities are often observed in the subacute polyserositis stage of colibacillosis (Barnes *et al.*, 2008). Microscopically, first changes consist of edema and heterophil infiltration. Macrophages are seen from 12 hours after inoculation and become the predominant inflammation cells, with giant cells along margins of necrotic areas. Lesions are further characterized by fibroplast proliferation and accumulation of vast numbers of necrotic heterophils in caseous exudate, in which numerous *E. coli* bacteria are present (Barnes *et al.*, 2008).

O-serotyping is the most frequently used typing method for diagnostic purposes and therefore often used for APEC description. *E. coli* serotypes mostly identified in disease outbreaks are O1, O2 and O78 (Barnes *et al.*, 2008; Dziva and Stevens, 2008). However, these serotypes can also be recovered from the faeces of healthy birds (Rodriguez-Siek *et al.*, 2005), reinforcing the suggestion that the intestinal tract may be an important natural reservoir for APEC and that predisposing factors may be required to produce disease.

1.2.2.2. Virulence associated factors in APEC

As mentioned before, the respiratory tract is a significant route of entry into the host, whilst the intestinal tract has been reported to be a reservoir for both pathogenic and non-pathogenic strains. APEC have shown to express several adhesins (fimbrial or non fimbrial) that could enhance preferential colonization of different sites. Colonization of multiple organs occurs in birds that survive septicaemia (Dziva and Stevens, 2008).

How APEC reaches the bloodstream is not yet clarified. Whether there is a direct invasion after damage of the respiratory epithelia or APEC gain entry following uptake by macrophages is unknown (Dziva and Stevens, 2008). In O2 strains, the adhesin *ibeA* seems to

play a role (Germon *et al.*, 2005). However, *ibeA* is also detected in APEC O78 from healthy birds (Rodriguez-Siek *et al.*, 2005).

Although tending to be less toxic than mammalian pathogenic *E. coli*, APEC has been shown to produce several toxins: a vacuolating autotransport toxin, enterohaemolysin, cytotoxic necrotic factor 1, cytolethal distending toxin and cytotoxin designated $VT2_y$ (Barnes *et al.*, 2008; Dziva and Stevens, 2008). In addition, APEC posses genes that encode iron acquisition mechanisms (such as aerobactin, yersiniabactin, *sit*, and *iro* systems) to sequester iron from the body fluids. These genes are considered pivotal for virulence and are significantly less common in commensal *E. coli* strains (Rodriguez-Siek *et al*., 2005). Also believed to play an important role in APEC virulence are serum resistance mechanisms protecting against complement-mediated lysis and opsonophagocytosis. Resistance is related to structural factors such as a smooth LPS layer or a particular LPS type, certain outer membrane proteins and specific capsule types (Barnes *et al.*, 2008; Dziva and Stevens, 2008). Finally, other mechanisms that have been reported are biofilm formation (Barnes *et al.*, 2008) and virulence gene regulators (Dziva and Barnes, 2008).

It is generally believed that respiratory colibacillosis is a secondary disease. However, having acquired all these genes that encode virulence factors suggests that certain clones of APEC may be especially adapted to a life as a pathogen (Rodriguez-Siek *et al.*, 2008). Vandekerchove *et al.* (2005) observed that certain virulence associated traits were significantly more prevalent in APEC isolates from colibacillosis outbreaks with high mortality. Nevertheless, a virulence factor existing in APEC but not detected in faecal *E. coli* strains from clinically healthy poultry, has not yet been identified (Barnes *et al.*, 2008).

1.2.2.3. Diagnosis, treatment and prevention of respiratory colibacillosis

The clinical signs and the presence of the typical macroscopic lesions are a first indication for respiratory colibacillosis. Isolation of APEC from the affected organs on Mac Conkey agar, eosin-methylene blue or drigalki agar confirms the diagnosis. Further identification is based on biochemical tests. Slide agglutination tests can be performed to verify the serotype of the strain. Recently, a multiplex PCR has been developed to detect a specific constellation of 6 virulence genes (*sitA*, *iroN*, *hlyF*, *iss*, *iutA*, and *etsA*) that appear to characterize virulent strains identified in >74% of APEC isolates, but occur infrequently in commensal strains (Barnes *et al.*, 2008).

Respiratory colibacillosis is mainly treated with antimicrobials, but APEC isolates are frequently resistant to one or more drugs (Barnes *et al.*, 2008). Avoiding the introduction of

pathogens that promote APEC infection in the turkeys, vaccinating the birds against respiratory pathogens and maintaining optimal environmental conditions are highly important in reducing the risk of respiratory colibacillosis. Measures that minimize dust and ammonia accumulation such as proper ventilation and good litter quality are fundamental since ammonia affects the clearance capacity of the respiratory tract and inhalation of dust contaminated with APEC is the most important source for infecting turkeys (Dho-Moulin and Fairbrother, 1999; Barnes *et al.*, 2008).

Some vaccines against APEC have been developed in turkeys. Effective inactivated vaccines have been produced (Arp, 1992; Trampel *et al.*, 1997), but they do not provide significant cross-protection against heterologous serogroups. Turkeys vaccinated with a *carAB* mutation of a virulent O2 serotype were protected against collibacillosis in a wildtype challenge model (Kwaga *et al.,* 1994). In addition, antiserum prepared in rabbits against iron-regulated outer membrane proteins of *E. coli* protected turkeys against mortality and reduced significantly macroscopic lesions and bacterial multiplication in the respiratory tract (Bolin and Jensen, 1987) (Barnes *et al.*, 2008). Although in Europe, a commercial vaccine is available for broiler breeders containing F11 fimbrial antigen and flagellar antigen, currently, no procedure has proved to be highly efficacious in commercial production and no product is used widely in the industry at the present time (Barnes *et al.*, 2008).

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CHAPTER 2: AIMS

AIMS

Respiratory disease in turkeys are induced by various viral and/or bacterial pathogens and are the cause of severe economic losses due to growth retardation, increased feed conversion rates, high medication costs, increased mortality rates and higher condemnation rates at slaughter. *Ornithobacterium rhinotracheale* and *Escherichia coli* are two bacterial species which play a significant role in the aetiology of the respiratory disease complex.

The current labelled dosage schedule of enrofloxacin for the treatment of bacterial respiratory disease caused by *O. rhinotracheale* or *E. coli* in turkeys and poultry advises a continuous drinking water treatment at 10 mg/kg BW for 5 successive days. Although the labelled dosage proves very efficacious, the regimen is regarded as cumbersome for many farmers, since the medicated drinking water has to be prepared freshly every day for five days. In addition, *in vivo* emergence of diminished susceptibility or even resistance to fluoroquinolones is commonly encountered in poultry amongst many bacterial species.

Over the last decade, dosage strategies have been developed for the fluoroquinolone antimicrobials which may increase their efficacy and may reduce the selection pressure for resistance. Nevertheless, controlled clinical studies which address such increased efficacy or minimal selection of resistance against *O. rhinotracheale* and *E. coli* infection have not been reported.

In view of the above, the aim of this research was to optimize the current enrofloxacin dosage regimen, by modifying

- the duration of the treatment
- and/or the daily administered enrofloxacin concentrations

in order to improve clinical and antimicrobial efficacy against respiratory disease caused by avian metapneumovirus (APV)/*O. rhinotracheale* and APV/*E. coli* infections in turkeys and to reduce the selection of less susceptible or resistant isolates.

CHAPTER 3: EXPERIMENTAL STUDIES

3.1. EFFICACY OF FOUR ENROFLOXACIN TREATMENT REGIMENS AGAINST EXPERIMENTAL INFECTION IN TURKEY POULTS WITH AVIAN METAPNEUMOVIRUS AND *ORNITHOBACTERIUM RHINOTRACHEALE*

3.2. EFFECT OF MULTIPLE AND SINGLE DAY ENROFLOXACIN MEDICATIONS AGAINST DUAL EXPERIMENTAL INFECTION WITH AVIAN METAPNEUMOVIRUS AND *ESCHERICHIA COLI* IN TURKEYS

3.3. THE EFFECT OF REDUCED TREATMENT TIME AND DOSAGE OF ENROFLOXACIN ON THE COURSE OF RESPIRATORY DISEASE CAUSED BY AVIAN METAPNEUMOVIRUS AND *ORNITHOBACTERIUM RHINOTRACHEALE*

3.1. EFFICACY OF FOUR ENROFLOXACIN TREATMENT REGIMENS AGAINST EXPERIMENTAL INFECTION IN TURKEY POULTS WITH AVIAN METAPNEUMOVIRUS AND *ORNITHOBACTERIUM RHINOTRACHEALE*

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Summary

Drinking water treatment with enrofloxacin is widely used to cure respiratory infections in turkeys. The current treatment regimen advises a five day treatment at 10 mg/kg body weight. Since enrofloxacin exerts a concentration-dependent activity it might be useful to provide the total dose of 50 mg/kg body weight in a single day treatment regimen. Therefore, we assessed whether single day treatment regimens with 50 mg/kg body weight were clinically equivalent to the advised multiple day treatment regimen with 10 mg/kg body weight for 5 days. For this purpose, five groups of 16 twenty-two day old turkeys were experimentally inoculated with avian metapneumovirus (APV) and *Ornithobacterium rhinotracheale* and subsequently treated in the drinking water with enrofloxacin, using either a single day treatment regimen at 50 mg/kg body weight during a 5, 10 or 20 hour period or a standard five day treatment regimen at 10 mg/kg body weight/ day for 20 hours. Although initially all dosage regimens cleared *O. rhinotracheale* from the trachea, four days after onset of treatment, *O. rhinotracheale* bacteria were re-excreted in the single day regimens but without worsening of the clinical symptoms. The five day treatment with 10 mg enrofloxacin/kg in turkeys provided the best results for the treatment of an *O. rhinotracheale* infection in turkeys by shortening the course and reducing the severity of clinical disease and by eliminating *O. rhinotracheale* from the respiratory tract without re-emergence. None of the used treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance.

Introduction

Respiratory disease induced by various viral and/or bacterial pathogens often affects turkeys during the rearing period causing severe economic losses due to growth retardation, increased feed conversion rates, increased mortality rates, higher condemnation rates at slaughter and high medication costs (van Empel & Hafez, 1999).

Avian metapneumovirus (genus *Metapneumovirus*) (APV) and *Ornithobacterium rhinotracheale* (*O. rhinotracheale*) play a significant role in the respiratory disease complex (van Empel *et al.*, 1996; Jirjis *et al.*, 2004). Avian metapneumovirus, causing disease in most turkey and chicken producing countries worldwide (Cook, 2000; Van de Zande, 2001; Jirjis *et al.*, 2002), also predisposes *O. rhinotracheale* to cause a secondary bacterial infection, thus aggravating the symptoms in the respiratory tract (Van Empel *et al*., 1996; Jirjis *et al.*, 2004; Marien *et al*., 2005; Marien *et al*., 2006). This double infection frequently occurs in the field where *O. rhinotracheale* is frequently isolated after APV infections (De Rosa *et al.*, 1996; El-Sukhon *et al.*, 2002) and is believed to play an important role in the respiratory problems in the turkey and chicken industry all over the world (Canal *et al.*, 2003; Van Loock *et al.*, 2005; Allymehr, 2006; Chansiripornchai *et al.*, 2007).

Pursuit of optimal environmental conditions such as good hygiene, adequate ventilation, low ammonia levels, optimal relative humidity levels and the prevention of simultaneous infections may reduce respiratory disease caused by *O. rhinotracheale* (van Empel and Hafez, 1999). However, clinical *O. rhinotracheale* related disease requires antimicrobial treatment for animal welfare and to limit economic losses.

Enrofloxacin, a fluoroquinolone antimicrobial compound, acts by inhibiting DNA gyrase and topoisomerase IV, two enzymes involved in the bacterial DNA replication (Martinez *et al.*, 2006). Its killing rate has been shown to be concentration dependent and it exerts a post-antibiotic effect (Fera *et al.*, 2002, Wang *et al.*, 2003). When administered in the drinking water for several successive days, as currently labelled (10 mg/kg body weight (BW) for 3 to 5 days), enrofloxacin has proven its efficacy against *O. rhinotracheale* infections in turkeys (Marien *et al.*, 2006; Marien *et al*., 2007). Since fluoroquinolone antimicrobials exert a concentration-dependent activity (Baggot, 2000; Prescott & Walker, 2000), in this study the hypothesis was tested whether a single daily treatment with the same total dose of 50 mg/kg BW would be as efficacious against *O. rhinotracheale* induced respiratory disease as a five daily treatment of enrofloxacin at 10 mg/kg BW. In addition, single day dosing would also improve user convenience by reducing the workload (preparing medicated water every day during treatment) for the farmer. The effect of single day drinking water treatments on the

course of a mixed APV/*O. rhinotracheale* infection has never been examined. Therefore, single-day-dosing treatments of the same total enrofloxacin dose, administered over a period of 5, 10 or 20 hours per day were compared to a continuous enrofloxacin dosage to turkeys experimentally infected with APV and *O. rhinotracheale.*

Materials and Methods

Turkeys

Eighty specified pathogen-free (SPF) turkeys (AFSSA, Ploufragan, France), hatched at our facilities, were used in this study. The birds were housed on litter in separate isolation rooms with HEPA-filtered air, had free access to feed and water and received 20 hours of light per day. At two weeks of age, the sera of the birds were shown to be free of maternally-derived antibodies to APV and *O. rhinotracheale* by means of commercially available ELISA-kits (Biochek, Gouda, the Netherlands). Tracheal swabs from all birds were examined for the presence of *O. rhinotracheale* and proved to be negative.

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al.*, 1998). The virus stock had a titre of 5.5 $\log_{10}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacteria

The *O. rhinotracheale* type strain LMG 9086^T, originally isolated from a turkey with a respiratory tract infection, was used. The strain was serotyped as type A in an agar gel precipitation test (Hafez & Sting, 1999), kindly performed by Professor H. M. Hafez (Institute of Poultry Diseases, Free University of Berlin, Germany). The minimum inhibitory concentration (MIC) of enrofloxacin for the strain was determined at $\leq$ 0.03 µg/ml and the strain was stored at -70°C. The organism was cultured for 48 h at 37°C on Columbia agar (Oxoid LTD, Basingstoke, Hampshire, England) with 5% sheep blood in a 5% CO_2 atmosphere. The *O. rhinotracheale* bacteria were transferred into brain heart infusion (BHI) broth (Oxoid) for 24 h at 37°C with agitation. The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in phosphate-buffered saline (PBS) followed each time

by five minutes of centrifugation at 3,000 rpm at 4°C. The resulting pellet was resuspended in PBS to obtain a final concentration of 8 $\log_{10}$ colony-forming units (cfu)/ml. Confirmation of the number of cfu/ml was done by inoculating ten-fold dilutions in PBS on sheep blood agar and counting the number of colonies.

Experimental design

Eighty SPF turkeys were randomly divided into five groups and subsequently infected with APV at twenty-one days of age. Three days later, the turkeys were inoculated with *O. rhinotracheale*.

All the birds of the respective groups were inoculated oculonasally with a dose of 4.4 $\log_{10}CD_{50}$ of APV and a dose of 8.5 $\log_{10}$ cfu of *O. rhinotracheale* by dividing a total of 250 µl inoculum equally over the nostrils and eyes.

Four groups received antimicrobial treatment by medicating the drinking water. Group E10-5D received 10 mg enrofloxacin (Baytril 10% oral solution)/kg body weight (BW) per day for 5 days, group E50-20H obtained 50 mg enrofloxacin/kg BW over a 20 h period, group E50-10H 50 mg enrofloxacin/kg BW over a 10 h period and group E50-5H 50 mg enrofloxacin/kg BW over a 5 h period. Control group C remained untreated. Enrofloxacin was administered 24 h after *O. rhinotracheale* inoculation. In every group, water uptake was measured on a daily basis from three days before until the end of the treatment periods. Additionally, all animals were weighed immediately before APV inoculation and again before *O. rhinotracheale* inoculation. On the basis of these data, the antimicrobial concentrations to be administered in the water and the actually received dosages were calculated. Water samples from the medicated drinking water were collected daily and stored at 4°C, protected from light, to determine the actual amount of enrofloxacin using turbulent flow chromatography/tandem mass spectrometry (Krebber, 2003).

All birds were examined clinically on a daily basis throughout the experiment until they were sacrificed. The clinical symptoms were divided into lower respiratory tract and upper respiratory tract symptoms. Dyspnoea was regarded as a sign of lower respiratory distress. The clinical signs of the upper respiratory tract (nasal exudates, swollen sinuses, frothy eyes) were scored as indicated in Table 1. The mean clinical score was calculated for each experimental group.

Table 1. Scoring system for quantification of clinical signs (based on Van de Zande *et al.*, 2001)

Score	Clinical signs
0	Absence of clinical signs
1	Clear nasal exudate
2	Turbid nasal exudate
3	Nasal exudate with mildly swollen infra-orbital sinuses
4	Nasal exudate with extremely swollen sinuses
5	Nasal exudate with extremely swollen sinuses and frothy eyes
6	Death

Tracheal swabs were taken from all birds 2 days post viral infection (pvi) for titration of APV.

Tracheal swabs were also collected on 1, 2, 4, 6, 8, 10 and 15 or 16 days post bacterial infection (pbi) from all groups for titration of *O. rhinotracheale.* The tracheal swabs were collected using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc.**,** Corona, USA) and placed in transport medium. The latter consisted of 1 ml PBS for bacteriological examination which was supplemented with 10% fetal calf serum (Gibco, Invitrogen Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco) for virus titration. Processing occurred as described below.

Five birds of each group were sacrificed at 6 days pbi. Equal numbers of the remaining birds of the 5 groups were sacrificed at 15 or 16 days pbi. Euthanasia was performed by intravenous injection with 0.3 ml/kg BW of T61 (Intervet, Belgium). The birds were necropsied and examined for the presence of gross lesions.

For the five birds of each group sacrificed at 6 days pbi, samples of the turbinates, trachea and lungs were collected for *O. rhinotracheale* examination. A 10% tissue suspension in PBS was prepared from these samples. Air sacs, pericardium and liver were sampled with cotton swabs for *O. rhinotracheale* re-isolation. All samples were processed immediately after collection as described below.

For the remaining birds sacrificed at 15 and 16 days pbi, turbinates, trachea, lungs, air sacs, liver and pericardium were sampled with cotton swabs for *O. rhinotracheale* re-isolation.

The birds were weighed the day before the APV infection, the day before the *O. rhinotracheale* infection and subsequently on 15 or 16 days pbi.

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2007/1).

Virological and bacteriological titration of tracheal swabs

The viral titre in $\log_{10}CD_{50}$ per ml and the number of cfu of *O. rhinotracheale* per ml were determined, using procedures described in Marien *et al.* (2005). In addition, in order to detect *O. rhinotracheale* isolates with reduced susceptibility to enrofloxacin, samples were also inoculated on sheep blood agar supplemented with 5 µg/ml gentamicin, 5 µg/ml polymyxin and 0.25 µg or 2 µg enrofloxacin/ml starting from 4 days pbi.

Bacteriological examination of tissue suspensions and swabs

From samples of the turbinates, trachea, and lungs, the cfu of *O. rhinotracheale* was determined in duplicate by incubating undiluted tissue suspensions and tenfold serial dilutions of tissue suspensions on sheep blood agar supplemented with 5 µg/ml gentamicin and 5 µg/ml polymyxin. After 24-48 h of incubation at 37°C in a 5% CO_2 atmosphere, viable counts were performed and the number of cfu/ml tissue suspension calculated.

The swabs taken from the air sacs, pericardium and liver from the 25 birds sacrificed at 6 dpbi and the swabs from the turbinates, trachea, lungs, air sacs, pericardium and liver from the birds sacrificed at 15 and 16 dpbi were inoculated on sheep blood agar supplemented with 5 µg/ml gentamicin and 5 µg/ml polymyxin. After 24-48 h of incubation at 37°C in a 5% CO_2 atmosphere, the agar was examined for the presence of *O. rhinotracheale* colonies.

Statistical analyses

Tracheal titres of *O. rhinotracheale* were analysed for all birds using the measurements between 0 and 6 days pbi and subsequently for the birds that were not euthanized using the measurements between 0 and 9 days pbi. The clinical scores and weight were analysed only for animals that were not euthanized at 6 days pbi.

The average over time of the clinical score and tracheal swabbing was analysed using a fixed-effects model with the treatment group as fixed effect.

Weight gain was compared at the end of the trial using a fixed-effects model.

O. rhinotracheale titres from the turbinates, trachea and lung of the animals, euthanized at 6 days pbi were compared between the five treatment groups with the Kruskall-Wallis test, whereas the presence of *O. rhinotracheale* in the airsacs, liver and pericardium of the animals,

euthanized at 6 days pbi were compared between the five treatments with the Fisher's exact test.

All tests were performed at a global 5% significance level and the pairwise comparisons were tested at an adjusted significance level using Bonferroni's multiple comparisons technique. (Agresti, 2002)

Results

Antimicrobial uptake

The actual dose for the single day medication periods was 40.8 mg/kg for group E50-5H, 47.8 mg/kg for group E50-10H and 72.5 mg/kg for group E50-20H. The mean actual daily dose for group E10-5D for the total medication period was 10.4 mg/kg (range 8.61-12.56 mg/kg).

Clinical signs

No animals died due to the APV/ *O. rhinotracheale* infections.

The mean clinical scores calculated for each group are presented in Figure 1.

Signs of upper respiratory distress were seen in all groups starting from 1 day pbi with the exception of 2 birds (one from group E10-5D and one from group E50-20H), which showed already clear nasal exudates shortly after APV infection.

Using the average of the clinical scores from 0 to 9 days pbi, there was a significant difference between the 5 treatments ($P = 0.0176$). The control group had the highest total score, followed by group E50-10H, group E50-5H, group E50-20H and group E10-5D. From the pairwise comparisons, only group E10-5D differed significantly from the control group.

Applying the 5 day dosing regimen, the clinical signs disappeared 4 days earlier than in the untreated group. In groups E50-20H and E50-5H, clinical signs disappeared 2 and 3 days earlier than in the control group.

Thus, compared with the untreated control group, clinical signs were significantly reduced only by a multiple day drinking water treatment with enrofloxacin 10 mg/kg body weight for 5 successive days.

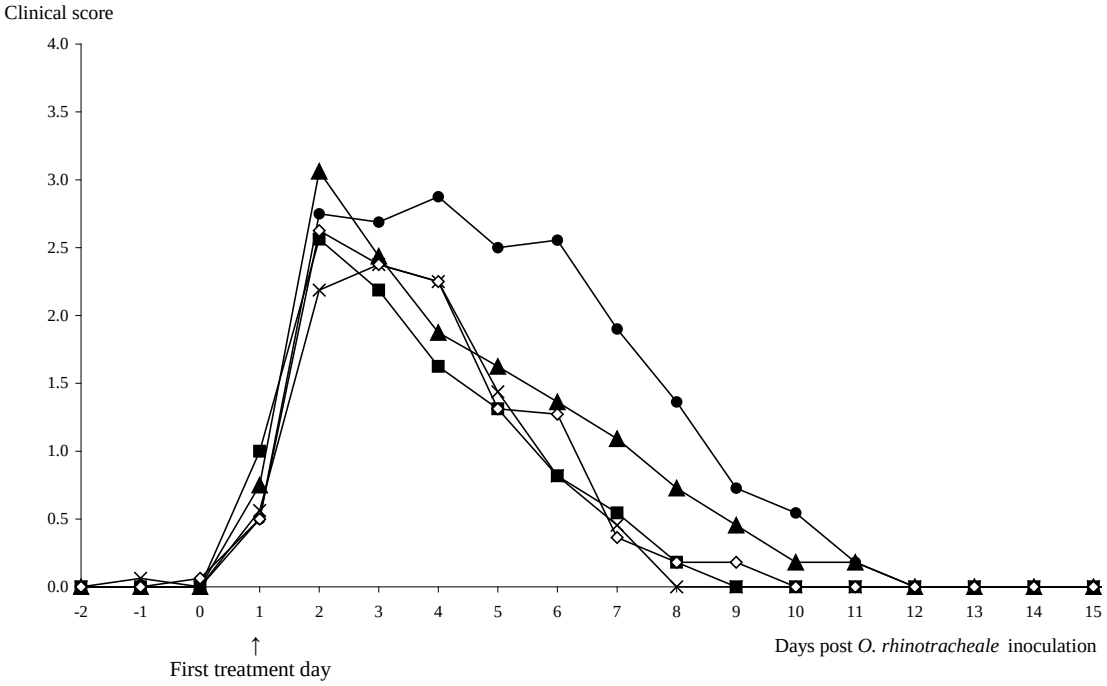

Figure 1. Mean clinical scores in turkeys, inoculated with APV followed by *O. rhinotracheale* three days later, and treated with 10 mg/kg/day enrofloxacin for 5 successive days (group E10-5D X), 50 mg/kg enrofloxacin for 20 hours (group E50-20H ◊), 50 mg/kg enrofloxacin for 10 hours (group E50-10H ▲), 50 mg/kg enrofloxacin for 5 hours (group E50-5H ■), or left untreated (●).

Macroscopic findings

No macroscopic lesions were found in group E10-5D. The severity of the lesions in the control group and the single day treatment groups were similar, although the highest lesion incidence was seen in the control group. Most birds suffered from sero- to seromucous exudate in the upper respiratory tract.

In groups E50-5H and E50-20H, pneumonia was present in 2 birds of each group and 1 bird in group E50-10H suffered from pneumonia and airsacculitis. In the control group pneumonia was present in 4 animals of which 2 also had airsacculitis.

The incidence of macroscopic lesions in the different treatment groups is shown in Table 2. Significant differences between treatments were observed for rhinitis (P = 0.0122) and sinusitis (P = 0.015). Inflammation of nostrils and sinuses was absent in group E10-5D, observed 4 times in group E50-5H, 8 times in group E50-20H and the control group and 9 times in group E50-10H.

Table 2. Incidence of macroscopic lesions at 6 days post bacterial infection and at 15 or at 16 days post bacterial infection of turkeys inoculated with APV followed by *O. rhinotracheale* three days later and treated with different enrofloxacin dosage schemes: 50 mg/kg for 5h (E50-5H), 50 mg/kg for 10h (E50-10H), 50 mg/kg for 20h (E50-20H), 10 mg/kg/day for 5 successive days (E10-5D) or no treatment (C).

Necropsy days pbi	Lesion type	Control	E50-5H	E50-10H	E50-20H	E10-5D
6	rhinitis (nostrils, conchae)	4/5 (*)	3/5	5/5	4/5	0/5
	sinusitis	4/5	1/5	4/5	4/5	0/5
	tracheitis	0/5	0/5	1/5	0/5	0/5
	pneumonia	4/5	2/5	1/5	2/5	0/5
	airsacculitis	2/5	0/5	1/5	0/5	0/5
	All lesions	14/25 (56%)	6/25 (24%)	12/25 (48%)	10/25 (40%)	0/25 (0%)
15 or 16	rhinitis (nostrils,conchae)	0/11	0/11	0/11	0/11	0/11
	sinusitis	0/11	0/11	0/11	0/11	0/11
	tracheitis	0/11	0/11	0/11	0/11	0/11
	pneumonia	8/11	4/11	6/11	7/11	1/11
	airsacculitis	2/11	0/11	0/11	0/11	0/11
	All lesions	10/55 (18%)	4/55 (7%)	6/55 (11%)	7/55 (13%)	1/55 (2%)

(*) number of positive observations/total number of observations

From the 11 birds that were necropsied per group at 15 and 16 days pbi, lung lesions were seen in 8 birds from the control group. In group E50-20H, group E50-10H and group E50-5H respectively 7, 6 and 4 turkeys showed lung lesions, whereas in group E10-5D lung lesions were seen in only 1 bird, with a significant difference between treatments (P = 0.0219). (Table 2).

Virological titration of tracheal swabs

2 Days post viral infection APV was recovered from tracheal swabs of every bird. Mean titres were 4.6 $\log_{10}CD_{50}$/ml, 4.3 $\log_{10}CD_{50}$/ml, 4.3 $\log_{10}CD_{50}$/ml, 4.7 $\log_{10}CD_{50}$/ml and 4 $\log_{10}CD_{50}$/ml for groups E10-5D, E50-20H, E50-10H, E50-5H and the control group respectively.

Bacteriological titration of tracheal swabs

The results of the *O. rhinotracheale* titrations of the tracheal swabs are shown in Figure 2.

After the first treatment day, *O. rhinotracheale* was hardly isolated out of turkeys treated with enrofloxacin. Only one turkey from group E50-10H showed very low *O. rhinotracheale* titres. This in contrast to 14 animals from the untreated control group. From 4 days pbi (group E50-5H) and 6 days pbi (group E50-10H and group E50-20H) onwards, however, *O. rhinotracheale* was isolated from birds from the single day enrofloxacin treatment groups, whereas in the five day enrofloxacin treatment group *O. rhinotracheale* did not reappear. Using the average count in the fixed-effects model, there was a significant difference between the five treatments (P < 0.0001). For the significant pairwise comparisons between 0 and 9 days after infection see Table 3. All enrofloxacin treatment regimens reduced the *O. rhinotracheale* counts compared to the control group. The five day treatment resulted in a more pronounced reduction than the single day treatments of 10 or 20 hours and the single day treatment of 5 hours resulted in a more pronounced reduction than the single day treatment of 20 hours.

Titration of the tracheal swabs on enrofloxacin supplemented agar plates (i.e. 0.25 µg/ml agar and 2 µg/ml agar) did not show any growth of *O. rhinotracheale* at any time during the experiment.

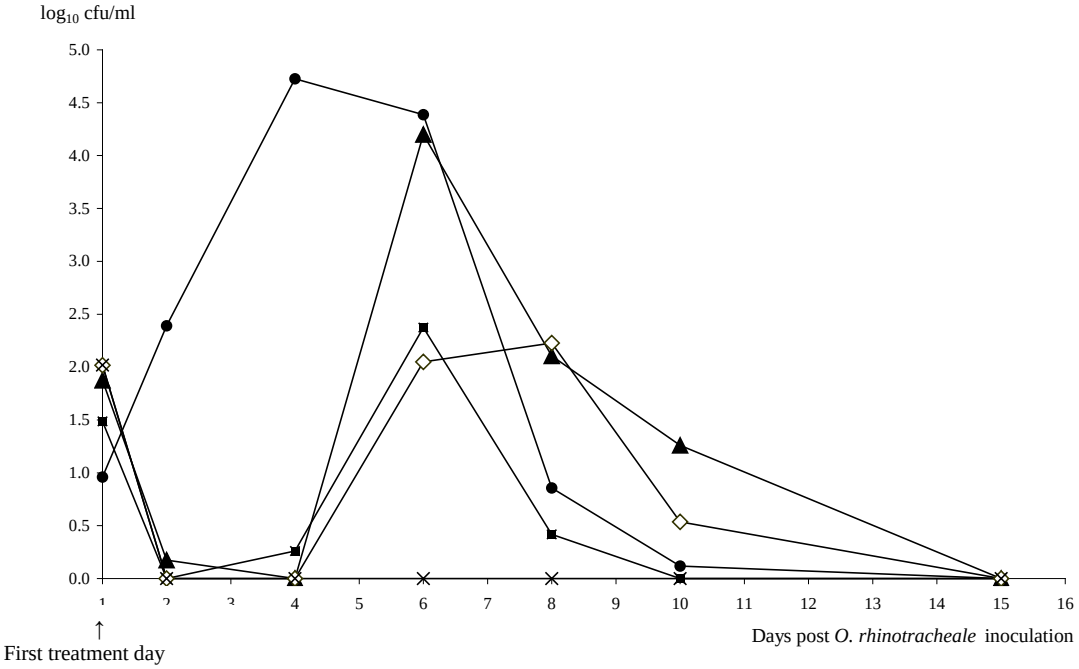

Figure 2. Mean *O. rhinotracheale* titres in tracheal swabs collected from turkeys, inoculated with APV followed by *O. rhinotracheale* three days later and treated with 10 mg/kg/day enrofloxacin for 5 successive days (group E10-5D X), 50 mg/kg enrofloxacin for 20 hours (group E50-20H ◊), 50 mg/kg enrofloxacin for 10 hours (group E50-10H ▲), 50 mg/kg enrofloxacin for 5 hours (group E50-5H ■), or left untreated (●).

Bacteriological examination of tissue suspensions and swabs

O. rhinotracheale was not isolated from any of the organs of the birds from group E10-5D. For the other groups, the mean *O. rhinotracheale* titres in the organs of the birds necropsied at 6 days pbi are shown in Table 3.

Significant differences between treatments were observed for *O. rhinotracheale* titres from the turbinates (P = 0.0023), trachea (P = 0.0177) and lungs (P = 0.0305). The control group showed the highest mean scores for the turbinates and lungs, followed by group E50-10H, group E50-5H, group E50-20H and group E10-5D. For the trachea, group E50-5H showed the highest mean score, followed by group E50-10H, the control group, group E50-20H and group E10-5D. The only pairwise significant difference was noted for the *O. rhinotracheale* titres in the turbinates between E10-5D and the control group. From the pericard, only three (the control group and group E50-10H) positive samples were obtained, and from the airsacs only one (group E50-10H). *O. rhinotracheale* was not recovered from any liver sample.

Table 3. Clinical scores and isolation of *O. rhinotracheale* out of tracheal swabs and organs (mean value *[standard error]*) of turkeys inoculated with APV followed by *O. rhinotracheale* three days later, and treated with different enrofloxacin dosage schemes: 50 mg/kg for 5h (E50-5H), 50 mg/kg for 10h (E50-10H), 50 mg/kg for 20h (E50-20H), 10 mg/kg/day for 5 successive days (E10-5D) or no treatment (C).

	Clinical Scores *	Isolation of *O. rhinotracheale* out of tracheal swabs*	Isolation of *O. rhinotracheale* at 6 days pbi (log_{10}cfu/g tissue)		
	Mean clinical scores	Mean Log_{10} cfu/ml	Turbinates	Trachea	Lungs
Group					
C	1.90 *[±1.06]* ^{a}	2.24 *[±1.94]* ^{a}	6.0 *[±1.16]* ^{a}	4.9 *[±2.19]* ^{a}	2.9 *[±1.86]* ^{a}
E50-5H	1.14 *[±0.8]* ^{ab}	0.76 *[±0.96]* ^{cd}	3.9 *[±1.16]* ^{ab}	5.7*[±1.46]* ^{a}	1.9 *[±1.73]* ^{a}
E50-10H	1.44 *[±0.94]* ^{ab}	1.6 *[±1.54]* ^{b}	4.7 *[±2.62]* ^{ab}	5.6 *[±2.49]* ^{a}	2.1 *[±1.95]* ^{a}
E50-20H	1.22 *[±1.00]* ^{ab}	1.14 *[±1.07]* ^{bc}	1.1 *[±2.37]* ^{ab}	3.2 *[±3.13]* ^{a}	0.00 ^{a}
E10-5D	1.12 *[±0.96]* ^{b}	0.34 *[±0.82]* ^{d}	0.00 ^{b}	0.00 ^{a}	0.00 ^{a}

^{abcd} treatments sharing the same superscript do not differ significantly from each other at the 5% global significance level
* from 0 to 9 days post bacterial inoculation (dpbi)

During the necropsies at days 15 and 16 pbi, *O. rhinotracheale* was recovered from the lungs of only one turkey from group E50-10H. *O. rhinotracheale* was not isolated from any other organ in any other bird.

Weight

After correction for gender, no significant differences (P = 0.65) were observed between the treatment groups.

Discussion

The incidence and severity of the clinical signs and macroscopic lesions, as well as the extent of the *O. rhinotracheale* tracheal and organ colonization, were reduced to the highest degree using the five day drinking water medication with enrofloxacin at a dose of 10 mg/kg BW. The effect of the three single day regimens, although significant, was less pronounced compared to the five day treatment. Hence, the single day dosage regimens are not a useful valuable alternative to the labelled dosing regimen. Five day treatment of *O. rhinotracheale* associated respiratory disease in turkeys seems therefore recommended. The current findings concur with the results obtained by Marien *et al.* (2006, 2007). The success of the high efficacy level of enrofloxacin is supported by several pharmacokinetic studies performed in turkeys (Heinen *et al.*, 1997; Dimitrova *et al.*, 2006; Fraatz *et al.*, 2006). According to these studies, a dose of 10 mg/kg enrofloxacin given orally at 24-h intervals, results in the establishment of a steady state concentration in the serum and respiratory tract that is bactericidal for *O. rhinotracheale* organisms with a MIC of 0.03 µg/ml. Each of the enrofloxacin dosage regimens applied, was able to clear the trachea from *O. rhinotracheale* after infection, but four days after the start of the treatment, the bacteria were re-isolated in every single-day-treated animal group. This re-excretion did not worsen the clinical signs. A plausible explanation would be that in the three single-day-dosing regimens not all birds ingested an sufficient amount of the medicated drinking water during the limited time that enrofloxacin was available due to competitive behaviour at the drinkers. This would thus result in lower enrofloxacin levels in the plasma and the respiratory tissues of subordinate birds. Possible sources for the re-emergence of *O. rhinotracheale* in the trachea could be either environmental recontamination or endogenous tracheal re-colonization. It was hypothesized that higher plasma and respiratory tissue concentrations, subsequent to these higher dosages, would exert a better concentration-dependent killing. However reduction of

tracheal *O. rhinotracheale* multiplication was similar to the reduction with the 5 day treatment and re-excretion was only seen in the single day treatments. This indicates that the duration of treatment at the appropriate dosage is as important for clinical efficacy as the size of the dose as such.

Acquired quinolone resistance is commonly encountered and is the consequence of stepwise mutations in the *gyr* and *par* genes (Barnard & Maxwell, 2001; Hooper, 2001). This means that after single step mutations the bacteria will become less-susceptible or even resistant after developing supplementary mutations. In this study, however, bacterial clones with reduced susceptibility or resistance were not detected in any of the enrofloxacin treated groups. This supports pharmacokinetic findings that, when enrofloxacin is used according to labelled directions for animal use, it minimizes the risk of selection for bacterial resistance.

Antimicrobial treatment is needed for reasons of animal welfare and for control of infectious diseases. With antimicrobial use in commercial birds under scrutiny it should be emphasized that antibiotics should always be used rationally and carefully to preserve the efficacy of these therapeutic agents. This means that enrofloxacin should only be used where a bacterial infection is known or suspected to be present as determined by direct demonstration of the infection or from the clinical data and that sensitivity testing of enrofloxacin should be conducted prior to its use (Prescott, 2000). It is also important to remember that even the best treatment programs can never replace good management. The positive effects of strict hygiene, effective biosecurity and sound flock management practices cannot be understated (Tanner, 2000).

In conclusion, all enrofloxacin treatment regimens reduced *O. rhinotracheale* multiplication in the respiratory tract tissues (turbinates, trachea and lung). Although initially the single day dosage regimens totally cleared *O. rhinotracheale* from the trachea, four days after onset of treatment *O. rhinotracheale* bacteria were re-excreted but without worsening of the clinical signs. The five day treatment with 10 mg enrofloxacin/kg in turkeys provided the best results by shortening the course of clinical disease, by stopping *O. rhinotracheale* multiplication in the respiratory tract without re-multiplication, and by reducing macroscopic lesions. As no *O. rhinotracheale* grew on the enrofloxacin supplemented media, none of the used treatment regimens resulted in the selection of bacterial clones with reduced susceptibility to enrofloxacin.

Acknowledgements

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3.2. EFFECT OF MULTIPLE AND SINGLE DAY ENROFLOXACIN MEDICATIONS AGAINST DUAL EXPERIMENTAL INFECTION WITH AVIAN METAPNEUMOVIRUS AND *ESCHERICHIA COLI* IN TURKEYS

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Poultry Science (2009), 88, 2093-2100

Summary

Escherichia coli (*E. coli*) infections are a common cause of respiratory disease in turkeys. To control these respiratory infections in turkeys, drinking water treatment with enrofloxacin is widely used. The current treatment schedule advises a five day treatment at 10 mg/kg body weight. Since enrofloxacin exerts a concentration-dependent activity, it might be useful to provide this 50 mg/kg total dose in a single day treatment regimen. Therefore, we assessed whether a single day treatment schedule with 50 mg/kg body weight was clinically equivalent to the advised multiple day treatment schedule with 10 mg/kg body weight for five days. For this purpose, three groups of 17 twenty-two day old turkeys were experimentally inoculated with APV and three days later with *E. coli.* One group received 10 mg enrofloxacin/kg body weight in the drinking water for five successive days. The second group received 50 mg enrofloxacin/kg body weight for 20h in the drinking water. The third group was enclosed as an untreated positive control group. Both the multiple and the single day enrofloxacin treatment regimens reduced *E. coli* multiplication in the respiratory tract tissues (turbinates, trachea and lung) but the five day treatment with 10 mg enrofloxacin/kg in turkeys provided the best results by shortening the course of clinical disease, by eliminating *E. coli* from the respiratory tract without re-multiplication, and by reducing macroscopic lesions. The efficacy of the single day treatment did not equal that of the five day treatment, possibly by not eliminating *E. coli* from the respiratory organs, which made it possible for the remaining bacteria to re-emerge in those organs. None of the used treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance.

Introduction

Enrofloxacin, a fluoroquinolone antimicrobial compound, acts by inhibiting DNA gyrase and topoisomerase IV, two enzymes involved in bacterial DNA replication (Martinez *et al.*, 2006). When administered in the drinking water for several successive days at 10 mg/kg body weight, enrofloxacin has proven its efficacy against several bacterial pathogens causing respiratory disease in turkeys like *Escherichia coli* (*E. coli*) (Marien *et al.*, 2007; Gautrais and Copeland, 1997), *Ornithobacterium rhinotracheale* (Marien *et al.*, 2006; Marien *et al.*, 2007) and *Pasteurella multocida* (Hafez *et al*., 1992; Gautrais and Copeland, 1997). Since the killing rate of enrofloxacin has been shown to be concentration dependent and exerts a post-antibiotic effect (Prescott and Walker, 2000), it is hypothesized that higher plasma and respiratory tissue concentrations, due to a higher dosage, could succeed in a better killing of bacteria. Therefore a single day treatment with the same total dose of 50 mg/kg body weight could be as efficacious as the currently labelled dose of 10 mg/kg body weight for 5 continuous days.

Colibacillosis is generally agreed to be the most common infectious bacterial disease of poultry. It refers to any localized or systemic infection caused entirely or partly by avian pathogenic *E. coli* (APEC), including colisepticemis, coligranuloma, swollen-head syndrome, air sac disease, venereal colibacillosis, coliform cellulitis, peritonitis, salpingitis, orchitis, turkey osteomyelitis, yolk sac infection, panophtalmitis and enteritis (Barnes *et al.*, 2008). These various forms of infection are responsible for severe economic losses due to growth retardation (Russel, 2003) and very high condemnations rates at slaughter (Yogaratnam, 1995; Jakob *et al.*, 1998; Dho-Moulin and Fairbrother, 1999).

Usually colibacillosis, affecting both chickens and turkeys, starts as a respiratory tract infection, evolving into a generalized infection, if unattended (Barnes *et al.*, 2008). In turkeys, susceptibility to colisepticemis is increased by avian metapneumovirus infection (APV) (Van de Zande *et al.*, 2001; Turpin *et al.*, 2002; Jirjis *et al.*, 2004), in chickens, by Infectious bronchitis virus and Newcastle disease virus (Ginns *et al*., 1998). In this study, a single-day-dosing treatment of the same total enrofloxacin dose was compared to a five day treatment in turkeys infected experimentally with APV and *E. coli*.

Materials and Methods

Turkeys

Fifty-one specified pathogen-free (SPF) turkeys (AFSSA, Ploufragan, France), hatched in our facilities and of mixed gender, were used in this study. The birds were housed on litter in separate isolation rooms with HEPA-filtered air, had free access to feed and water and received 20 hours of light per day. At two weeks of age the birds were shown to be free from maternally-derived antibodies to APV, by means of a commercially available ELISA (Biochek, Gouda, the Netherlands).

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al.*, 1998). The virus stock had a titer of 5.5 $\log_{10}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacteria

A Belgian *E. coli* isolate retrieved from turkeys with colibacillosis was used. It was identified and serotyped as O2:K1 by Dr. Flemming Scheutz (Statens Serum Institute, The International Escherichia and Klebsiella Centre, Copenhagen, Denmark). This strain has been used previously by Van de Zande *et al.* (2001) for establishing an *E. coli* infection in APV infected turkeys and was stored at -70°C. The minimum inhibitory concentration (MIC) of enrofloxacin for the strain was determined at $\leq$ 0.03 µg/ml. The organism was retrieved from the frozen suspension and cultured overnight at 37°C on MacConkey agar. The *E. coli* bacteria were transferred into Brain Heart Infusion (BHI) and cultured for 24 h at 37°C. The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in Phosphate Buffered Saline (PBS) followed each time by five minutes of centrifugation at 3,000 rpm at 4°C. The resulting pellet was re-suspended in PBS to obtain a final concentration of approximately 8 $\log_{10}$ cfu/ml. Confirmation of the number of cfu/ml was done by inoculating ten-fold dilutions in PBS on sheep blood agar and counting the number of colonies.

Experimental Design

Fifty-one SPF turkeys were randomly divided into three groups of 17 animals at twenty-one days of age. At twenty-two days of age the birds of all groups were inoculated with APV. Three days later the turkeys were inoculated with *E. coli*.

All the birds of the respective groups were inoculated oculonasally with a dose of 4.4 $\log_{10}CD_{50}$ for APV and a dose of 7.57 $\log_{10}$ cfu for *E. coli* by dividing a total of 250 µl inoculum equally over the nares and eyes.

All birds were examined clinically on a daily basis throughout the experiment until they were sacrificed. The clinical symptoms were divided into lower respiratory tract and upper respiratory tract symptoms. Dyspnea was regarded as a sign of lower respiratory distress. The clinical signs of the upper respiratory tract (nasal exudates, swollen sinuses, frothy eyes, possibly resulting in poor general condition and anorexia) were scored as described in Van de Zande *et al*. (2001). Briefly, the clinical condition of each bird was assigned a score from 0 (absence of clinical signs) to 3 (nasal exudate with mildly swollen infraorbital sinuses) to 6 (death).

All the birds were weighed the day before the APV infection, the day before the *E. coli* infection and subsequently on day 15 or 16 post bacterial infection (days pbi).

Two groups received antimicrobial treatment by medicating the drinking water. Group E10-5D received enrofloxacin 10 mg/kg for 5 days and group E50-20H enrofloxacin 50 mg/kg for 20h. Group C was enclosed as an untreated control group. The enrofloxacin (Baytril® 10% oral solution) treatments were administered 24h after *E. coli* inoculation. In every group, water uptake was noted on a daily basis from three days before treatment. Water uptake in each group during the different treatment periods was also noted. On the basis of these data, the antimicrobial concentrations to be administered in the water and the actually received dosages were calculated. Water samples from the medicated drinking water were collected daily and stored at 4°C, protected from light to determine the actual amount of enrofloxacin using turbulent flow chromatography/tandem mass spectrometry (Krebber, 2003).

Before *E. coli* inoculation, tracheal swabs from all birds of each experimental group were examined for *E. coli* O2:K1.

Tracheal swabs were also collected on 1, 2, 4, 6, 8, 10 and 15 or 16 days pbi from all groups for bacteriological titration of *E. coli.*

The tracheal swabs were collected using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc.**,** Corona, USA) and placed in transport medium. The latter consisted of 1 ml PBS for *E. coli* examination, supplemented with 10% fetal calf serum (Gibco, Invitrogen

Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco) for virus titration. Processing occurred as described below.

Five birds of each group were sacrificed at 6 days pbi. The remaining birds of each group were sacrificed 9 and 10 days after euthanasia of the first 5 birds, i.e. 15 and 16 days pbi, in similar ratios of the three groups. Euthanasia was performed by intravenous injection with an overdose (0.3 ml/kg bodyweight) of T61 (Intervet, Belgium). The birds were necropsied and examined for gross lesions.

For the five birds of each group sacrificed at 6 days pbi, samples of the turbinates, trachea and lungs were collected for *E. coli* examination. A 10% tissue suspension in PBS was made from these samples. Air sacs, pericardium and liver were likewise sampled with cotton swabs for *E. coli* analysis. All samples were processed immediately after collection as described below.

For the remaining birds sacrificed at 15 and 16 days pbi, turbinates, trachea, lungs, air sacs, liver and pericardium were sampled with cotton swabs for *E. coli* analysis. A detailed schedule of events is illustrated in Table 1.

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University EC 2007/01.

Virological and Bacteriological Titration of Tracheal Swabs

The viral titer in $\log_{10}CD_{50}$ per ml and the number of cfu *E. coli* per ml were determined. This was done using the procedures described in Marien *et al*. (2005), with the following addition to the protocol: for the isolation of *E. coli,* samples were inoculated on MacConkey agar and incubated for 24h at 37°C. Starting from 4 days pbi samples were also inoculated on MacConkey agar supplemented with 0.25 µg enrofloxacin/ml agar or 2 µg enrofloxacin/ml agar for the detection of *E. coli* isolates with reduced susceptibility to enrofloxacin.

The recovered *E. coli* isolates were serotyped by a slide-agglutination test using the antiserum against the *E. coli* O2:K1 strain (Veterinary Laboratories Agency, Surrey, United Kingdom).

Bacteriological Examination of Tissue Suspensions and Swabs

Samples of the turbinates, trachea and lungs were quantified for *E. coli* from the 15 birds sacrificed at 6 days pbi. The number of cfu of *E. coli* per ml tissue suspension was determined as described in Marien *et al*. (2005) with the following addition to the protocol: for the isolation of *E. coli,* samples were inoculated on MacConkey agar and incubated for 24h at 37°C.

The swabs taken from the air sacs, pericardium and liver from the 15 birds sacrificed at 6 days pbi and the swabs from the turbinates, trachea, lungs, air sacs, pericardium and liver from the birds sacrificed at 15 and 16 days pbi were inoculated onto MacConkey agar for *E. coli* isolation. After 24h of incubation at 37°C, the agar was examined for presence of *E. coli*.
The recovered *E. coli* isolates were serotyped by a slide-agglutination test using the antiserum against the *E. coli* O2:K1 strain (Veterinary Laboratories Agency).

Weight

As described above, the birds were weighed the day before the APV infection, the day before the *E. coli* infection and subsequently on 15 or 16 days pbi.

Statistical Analyses

First, analyses were based on all animals, using only results from 0 to 6 days pbi. The mean clinical score and tracheal titer of *E. coli* between 0 to 6 days pbi was determined for each turkey and used as response variable in a fixed-effects model with the treatment group as fixed effect. Second, analyses were based on turkeys that were not euthanized at day 5, using results between 0 to 9 days pbi.
The mean clinical score and tracheal titer of *E. coli* between 0 to 9 days pbi was determined for each turkey and used as response variable in a fixed-effects model with the treatment group as fixed effect.
Weight was compared at the end of the trial using a linear fixed effects model with gender and treatments as covariates.
All tests in the fixed-effects model were performed at a global 5% significance level with the P-values for the pairwise comparisons adjusted using Tukey's multiple comparisons technique.
E. coli titers from the turbinates, trachea and lung of the turkeys, euthanized at 6 days pbi were compared between the three treatment groups using the Kruskall-Wallis test, whereas the presence of *E. coli* in the airsacs, liver and pericardium of the turkeys, euthanized at 6 days pbi were compared between the three treatments using the Fisher's exact test. All these tests were performed at a global 5% significance level and the pairwise comparisons were tested at an adjusted significance level using Bonferroni's multiple comparisons technique.

Table 1. Schematic representation of the experimental events

Days after APV infection	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	...	18/19
Days after *E. coli* infection	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	…	15/16
Group E10-5D	W	APV		W	*E.coli*	TS E	TS E	E	TS E	E	TS *		TS		TS		TS/W *
Group E50-20H	W	APV		W	*E.coli*	TS E	TS		TS		TS *		TS		TS		TS/W *
Control group	W	APV		W	*E.coli*	TS	TS		TS		TS *		TS		TS		TS/W *

W: weighing of the turkeys
E: enrofloxacin treatment
day
APV: experimental infection with APV
TS: tracheal swabbing for titration
for *E. coli*
E. coli: experimental infection with *E. coli*
*: necropsy day

Results

Antimicrobial Uptake

The actual dose for the single day medication period was 51.3 mg/kg for group E50-20H. The mean actual daily dose for group E10-5D for the total medication period was 10.2 mg/kg (range 7.2-11.2 mg/kg). From this we can state that the consumed dose of enrofloxacin per group of birds was close to the target dose of 50 mg/kg and 10 mg/kg body weight.

Clinical Signs

No animals died due to the APV/ *E. coli* infections.

The mean clinical scores calculated for each group are presented in Figure 1.

Signs of upper respiratory distress were seen in all groups starting from 1 day post *E. coli* infection, with the exception of 2 birds from group E10-5D and 1 bird from the control group which showed already clear nasal exudates shortly after APV infection.

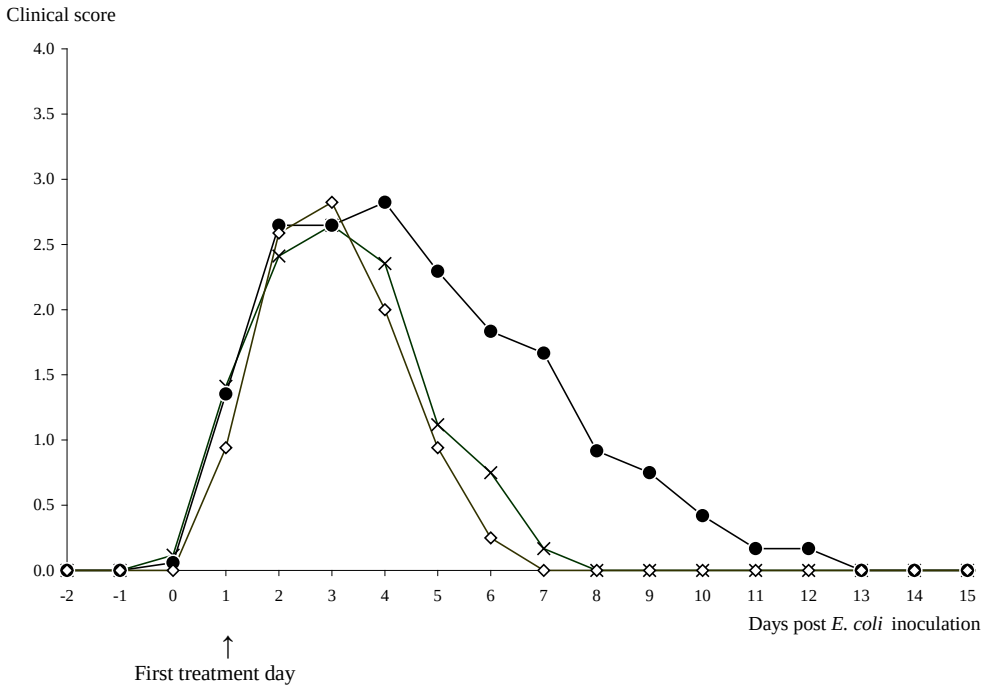

Figure 1. Mean clinical scores in turkeys, inoculated with APV followed by *E. coli* three days later, and treated with 10 mg/kg enrofloxacin for 5 days (group E10-5D x) or with 50 mg/kg enrofloxacin for 20 hours (group E50-20H ◊). Group C (●) was enclosed as an untreated positive control group. Enrofloxacin was administered in the drinking water, 24h after *E. coli* inoculation.

No significant differences between the three groups occurred for the mean clinical score between 0 to 6 days pbi (P = 0.1926). For the mean clinical score between 0 to 9 days pbi, there is a significant difference between the 3 treatments (P = 0.0064). The control group has the highest total score, followed by group E50-20H and group E10-5D. The treatment group E10-5D (P = 0.0243) and E50-20H (P = 0.0094) differed significantly from the control group. No significant difference was noted between the single and the multiple day treatment (P = 0.9226). In group E50-20H and group E10-5D the clinical symptoms for any of the animals disappeared respectively 5 and 6 days earlier than the control group.

Macroscopic Findings

The incidence of macroscopic lesions in the different treatment groups is illustrated in Table 2.

Only few macroscopic lesions, limited to the presence of rhinitis in 3 birds, were found in group E10-5D.

Most birds in group E50-20H and in the control group suffered from serous to seromucous exudate in the upper respiratory tract and both groups contained birds with lesions in the lower respiratory tract. However, the incidence of these lower respiratory tract lesions was much higher in the control group, in which three birds with pneumonia and 2 birds with airsacculitis lesions were observed. In the single day enrofloxacin treatment group only one bird showed lung lesions.

Table 2. Presence of macroscopic lesions at 6 days post bacterial infection (pbi) of turkeys inoculated with APV followed by *E. coli* three days later and treated with a multiple day enrofloxacin dosage regimen (10 mg/kg/day for 5 successive days (E10-5D)), a single day enrofloxacin dosage regimen (50 mg/kg for 20h (E50-20H)) or no treatment (C)

Lesion type	C	E50-20H	E10-5D
rhinitis (nostrils, turbinates)	4/5 (*)	3/5	3/5
sinusitis	0/5	2/5	0/5
tracheitis	0/5	0/5	0/5
pneumonia	3/5	1/5	0/5
airsacculitis	2/5	0/5	0/5
all respiratory lesions	9/25 (36%)	6/25 (24%)	3/25 (12%)

(*) number of positive observations/total number of observations

Necropsy at 15 and 16 days pbi of the remaining birds from the control group showed one bird with airsacculitis and another bird with rhinitis and pneumonia. No lesions were found in any turkey of the enrofloxacin treatment groups.

Virological Titration of Tracheal Swabs

Virological titration of tracheal swabs collected at 2 days post viral infection showed that APV was recovered from every bird. Mean titers were 4.6 $\log_{10}CD_{50}$/ml, 4.9 $\log_{10}CD_{50}$/ml and 4.9 $\log_{10}CD_{50}$/ml for group 10E-5D, group 50E-20H and the control group respectively.

Bacteriological Titration of Tracheal Swabs

The results of the *E. coli* titrations of the tracheal swabs are shown in Figure 2.

As from 1 day after bacterial infection, *E. coli* titers in the control group started to increase, resulting in maximal titers of 4.32 $\log_{10}$ cfu/ml at 6 days pbi after which bacterial numbers decreased to 0 at 15 days pbi.

After the first treatment day, i.e. at 2 days pbi, no *E. coli* was isolated out of any trachea in the five day enrofloxacin treatment group for the remaining course of the experiment. The single day enrofloxacin treatment highly reduced *E. coli* multiplication in the tracheae but was not able to obtain a permanent clearance of the infection. *E. coli* titers increased, starting from 4 days pbi with maximal titers of 1.20 $\log_{10}$ cfu/ml at 6 days pbi, a significantly lower number compared to that of the control group.

There is a significant difference between the three treatments ($P < 0.0001$) for the mean *E. coli* titers between 0 to 6 days pbi. The two treatment groups differ significantly from the control group ($P < 0.0001$).

There is also a significant difference between the three treatments ($P < 0.0001$) for the mean *E. coli* titer between 0 to 9 days pbi. The two treatment groups differ significantly from the control group ($P < 0.0001$). Furthermore, the five day treated group had significantly lower *E. coli* titers than the single day treated group ($P = 0.0053$).

Titration of the tracheal swabs on enrofloxacin supplemented agar plates (i.e. 0.25 µg/ml agar and 2 µg/ml agar) did not show any growth of less susceptible or resistant *E. coli* isolates at any time during the experiment.

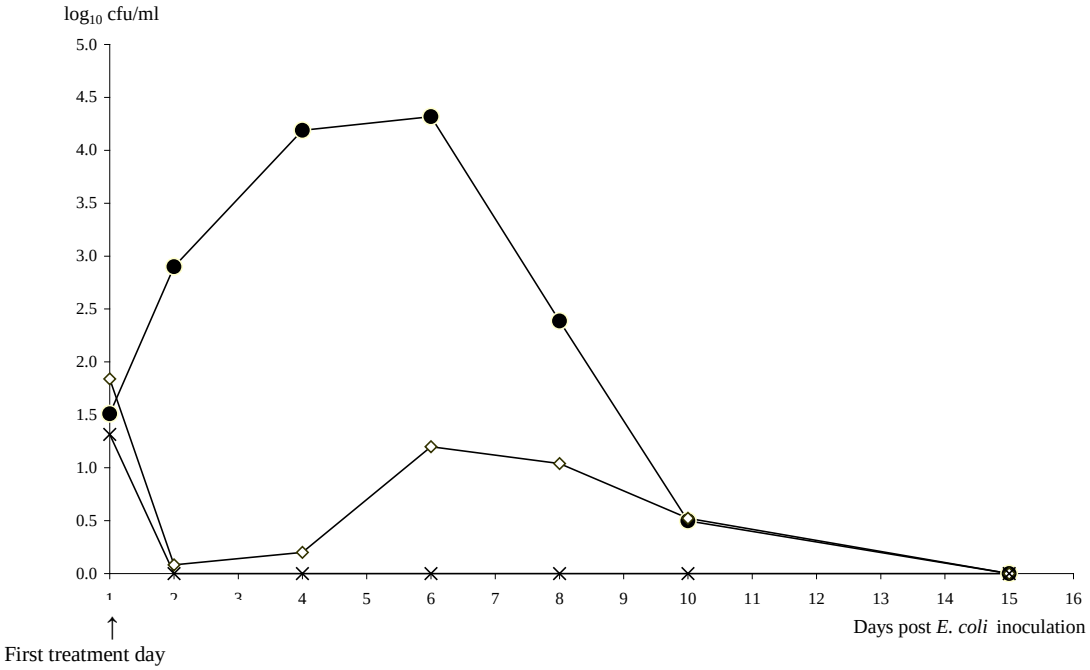

Figure 2. Mean *E. coli* titers in trachea mucus collected at different time points from turkeys, inoculated with APV followed by *E. coli* three days later, treated with 10 mg/kg enrofloxacin for 5 days (group E10-5D x) or with 50 mg/kg enrofloxacin for 20 hours (group E50-20H ◊). Group C (●) was enclosed as an untreated positive control group. Enrofloxacin was administered in the drinking water, 24h after *E. coli* inoculation.

Bacteriological Examination of Tissue Suspensions and Swabs

E. coli was never isolated from any of the organs of the birds from group E10-5D.

For the control group and group E50-20H, the various mean *E. coli* titers in the organs at 6 days pbi are shown in Table 3.

Significant differences between treatments were observed for *E. coli* titers from the turbinates, and tracheas. For these organs, the control group showed the highest mean scores, followed by group E50-20H and group E10-5D (lowest mean score). For the turbinates, a pairwise significant comparison was noted after Bonferroni correction between the control group and E10-5D. No pairwise significant differences were noted for *E. coli* titers of the trachea and no significant difference between treatments was noted for the titers of the lungs.

No *E. coli* was isolated from the air sacs, pericardium or liver in any bird.

During the final necropsy, *E. coli* was discovered in only one lung in a turkey from group E50-20H. In the control group, *E. coli* was isolated 3 times from turbinates, 1 time out from airsacs and one time from a trachea. No *E. coli* was isolated from any turkey of group E10-

5D. No significant difference between treatments was noted for the presence of *E. coli* in the organs during the final necropsy.

Table 3. Clinical scores and isolation of *E. coli* out of tracheal swabs and organs (mean value *(standard error)*) of turkeys inoculated with APV followed by *E. coli* three days later, and treated with a multiple day enrofloxacin dosage scheme (10 mg/kg/day for 5 successive days (E10-5D)), a single day enrofloxacin dosage scheme (50 mg/kg for 20h (E50-20H)) or no treatment (C)

	Clinical Scores *	Isolation of *E. coli* out of tracheal swabs*	Isolation of *E. coli* at 6 days pbi (log_{10}cfu/ml tissue suspension)		
	Mean clinical scores	Log_{10} cfu/ml	Turbinates	Trachea	Lungs
Group					
C	1.92 *(0.92)*	2.63 *(1.50)*	7.2 *(0.8)* [a]	3.7 *(2.1)* [a]	1.7 *(2.4)* [a]
E50-20H	1.19 *(1.14)* [a]	0.81 *(0.67)*	1.5 *(3.4)* [ab]	0.4 *(0.9)* [a]	0.00 *(0.00)* [a]
E10-5D	1.37 *(1.01)* [a]	0.22 *(0.54)*	0.0 *(0.00)* [b]	0.00 *(0.00)* [a]	0.00 *(0.00)* [a]

[a] treatments sharing a letter differ not significantly from each other at the 5% global significance level
* from 0 to 9 days post bacterial inoculation (pbi)

Weight

The mean weight gains measured in the after treatment period (i.e. from 1 day before *E. coli* infection until 15 or 16 days pbi) in the different turkey groups are 925 g for the control group, 968 g for group E50-20H and 1038 g for group E10-5D. Although no significant differences for weight gain are observed between the different groups (P = 0.4749) during the after-treatment period**,** there's a tendency, showing higher weight gains for longer treatment times (group E10-5D>group E50-20H> control group).

Discussion

The five day drinking water medication with enrofloxacin at a dose of 10 mg/kg body weight was the most successful in reducing the severity and incidence of the clinical signs and macroscopic lesions. *E. coli* tracheal and organ colonization were limited to the highest extent. Less pronounced, although significant, was the outcome of the single day treatment. As enrofloxacin exerts a concentration-depending activity (Prescott and Walker, 2000), it was hypothesized that higher plasma and respiratory tissue concentrations, due to a higher dosage, could succeed in a better killing of the *E. coli* bacteria. However, the single day treatment proved not as efficacious as the five day treatment regimen of 10 mg enrofloxacin/kg body weight in reducing *E. coli* multiplication in the respiratory organs. In contrast to the five day treatment, single day treatment did not achieve elimination of *E. coli* from the respiratory tract and the remaining bacteria re-emerged. This indicates that spread of the pathogen to susceptible animals could not be avoided using the single day treatment. The duration of the treatment at the appropriate dosage seems therefore as important for clinical efficacy as the size of the dose. The results obtained in this study, comparing a single and multiple treatment regimen, are highly similar to a previously performed study using similar enrofloxacin treatment regimens in turkeys infected experimentally with APV and *O. rhinotracheale* (Garmyn *et al.*, in press). Therefore, a single day dosage regimen is not a useful valuable alternative to the labelled dosage regimen in treating *E. coli* associated respiratory disease in turkeys and a five day enrofloxacin treatment regimen seems recommended.

Avian pathogenic *E. coli* are frequently found to be resistant to commonly used antibacterial agents as ampicillin, amoxycillin, tetracyclines, trimethoprim+sulphonamide and flumequine. Also, resistance to enrofloxacin is commonly encountered (Vandemaele *et al*., 2002). Acquired quinolone resistance is the consequence of stepwise mutations in the *gyr* and *par* genes. A first step mutation leads to reduced susceptibility after which successive mutations

possibly result in resistance (Barnard and Maxwell, 2001; Hooper, 2001). In this study, however, bacterial clones with reduced susceptibility or resistance were not detected in any of the enrofloxacin treated groups. Reduced susceptibility or resistance to enrofloxacin also did not occur when similar dosing regimens were tested previously on turkeys experimentally infected with *O. rhinotracheale* (Garmyn *et al.*, in press). This supports pharmacokinetic findings (Dimitrova *et al.*, 2006; Fraatz *et al.*, 2006) that, when enrofloxacin is used according labelled directions, the risk of selection for bacterial resistance is minimized.

In conclusion, both the multiple and the single day enrofloxacin treatment regimen reduced *E. coli* multiplication in the respiratory tract tissues (turbinates, trachea and lung), but the five day treatment with 10 mg enrofloxacin/kg in turkeys provided the best results by shortening the course of clinical disease, by eliminating *E. coli* from the respiratory tract without re-multiplication, and by reducing macroscopic lesions. The efficacy of the single day treatment did not equal that of the 5 day treatment, possibly by not eliminating *E. coli* from the respiratory organs, which made it possible for the remaining bacteria to re-emerge in those organs.

None of the used treatment schemes promoted the selection of bacterial clones with reduced susceptibility or resistance.

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3.3. THE EFFECT OF REDUCED TREATMENT TIME AND DOSAGE OF ENROFLOXACIN ON THE COURSE OF RESPIRATORY DISEASE CAUSED BY AVIAN METAPNEUMOVIRUS AND *ORNITHOBACTERIUM RHINOTRACHEALE*

Garmyn, A., Martel, A., Froyman, R., Ludwig, C., Nauwynck, H., Haesebrouck, F. and Pasmans, F.

Poultry Science (2009), 88, 2315-2323.

Summary

A dose titration and reduced duration medication study were performed to evaluate the current enrofloxacin treatment schedule in growing turkeys experimentally infected with avian metapneumovirus (APV) and *Ornithobacterium rhinotracheale* (*O. rhinotracheale*). Experimental groups of 17 four-week-old turkeys were first infected with avian metapneumovirus (APV) and three days later with *O. rhinotracheale*. Enrofloxacin treatment in the drinking water was started 24 h after *O. rhinotracheale* inoculation. In the dose titration study enrofloxacin doses of 5, 10 and 20 mg / kg body weight (BW) were administered for 5 successive days. In the reduced duration medication study, following enrofloxacin regimens were compared: 25 mg / kg BW / day on day 0 and day 2, 15 mg / kg BW / day on days 0, 2 and 4 and 10 mg / kg BW for 5 successive days. In both studies, all enrofloxacin treatments were equally efficacious, i.e. equally capable of shortening the course of clinical disease, eliminating *O. rhinotracheale* from the respiratory tract and reducing gross lesions. *O. rhinotracheale* bacteria were not recovered from any of the animals on enrofloxacin supplemented media, indicating that none of the used treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance to this antimicrobial agent. In conclusion, none of the alternative enrofloxacin treatment regimens yielded better results than the current prescribed treatment (i.e. 10 mg/kg BW for 5 successive days) of *O. rhinotracheale* infections in turkeys. However, the reduced duration of application would offer a less time-consuming and equally effective alternative.

Introduction

Respiratory disease induced by various viral and/or bacterial pathogens often affects turkeys during the rearing period causing severe economic losses due to growth retardation, increased feed conversion rates, increased mortality rates, higher condemnation rates at slaughter and high medication costs (van Empel and Hafez, 1999).

Ornithobacterium rhinotracheale (*O. rhinotracheale*) plays a significant role in the aetiology of the respiratory disease complex (van Empel *et al.*, 1996; Jirjis *et al.*, 2004). Although indications of primary pathogenicity exist (Van Veen *et al*., 2000; Sprenger *et al.*, 1998), the organism is mostly regarded as a facultative pathogen, aggravating the symptoms in the respiratory tract caused by viruses such as avian metapneumovirus (genus *Metapneumovirus*) (APV) (Van Empel *et al*., 1996; Jirjis *et al.*, 2004; Marien *et al*., 2005, 2006). This dual APV/ *O. rhinotracheale* infection frequently occurs in the field (De Rosa *et al.*, 1996; El-Sukhon *et al.*, 2002).

Pursuit of optimal environmental conditions such as good hygiene, adequate ventilation, low ammonia levels, optimal relative humidity levels and the prevention of simultaneous infections may reduce respiratory disease caused by *O. rhinotracheale* (van Empel and Hafez, 1999). In practice, *O. rhinotracheale* infections are primarily dealt with using antimicrobials in the drinking water. Enrofloxacin, a fluoroquinolone antimicrobial compound, is in Europe commonly used to cure respiratory infections in turkeys and has proven its efficacy against *O. rhinotracheale* infections, when administered in the drinking water for five successive days at a dose of 10 mg/kg body weight (BW), which is the labelled dosage (Marien *et al.*, 2006; Marien *et al*., 2007). This treatment regimen, however, is considered as cumbersome and time consuming by many farmers since the medicated drinking water has to be prepared each day during five days. This often hinders completion of the full treatment regimen. Other controlled clinical studies, addressing enrofloxacin efficacy against *O. rhinotracheale* infections, have not been reported. In addition, acquired resistance or reduced susceptibility of *O. rhinotracheale* against enrofloxacin is commonly encountered (Devriese *et al*., 1995, Malik *et al.*, 2003, Soriano *et al*., 2003, Van Veen *et al*., 2000) and more specific data are required to search for a therapeutic regimen that minimizes the risk of selecting antimicrobial resistant bacteria. In the present study, the efficacy of alternative enrofloxacin treatment regimens against *O. rhinotracheale* infections in experimentally infected turkeys was assessed. First the minimum effective dose of enrofloxacin in animals treated during five days was determined. In a second study, the efficacy of drinking water treatment with high doses of enrofloxacin, administered over a shorter period of time was determined.

In both experiments, the selection of *O. rhinotracheale* clones with reduced susceptibility or resistance was also assessed.

Materials and Methods

Turkeys

Commercial turkey poults (Moorgut Kartzfehn, von Kameke GmbH & Co, Bösel, Germany) were used in this study. The birds were housed on litter in separate isolation rooms with HEPA-filtered air, water and feed were available ad libitum, and received 20 hours of light per day. Before bacterial inoculation, tracheal swabs from all birds were examined for the presence of *O. rhinotracheale* and determined to be negative.

Virus

The APV strain A/T6/96 (subtype A) was used. The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande *et al.*, 1998). The virus stock had a titre of $10^{5.5}$ 50% ciliostatic dose (CD_{50})/ml after the third passage in tracheal organ cultures.

Bacteria

The *O. rhinotracheale* type strain LMG 9086^T, originally isolated from a turkey with a respiratory tract infection, was used. The strain was serotyped as type A in an agar gel precipitation test (Hafez and Sting, 1999), kindly performed by Professor H. M. Hafez (Institute of Poultry Diseases, Free University of Berlin, Germany). The minimum inhibitory concentration (MIC) of enrofloxacin for the strain was determined at $\leq$ 0.03 µg/ml and the strain was stored at -70°C. The organism was cultured for 48 h at 37°C on Columbia agar (Oxoid LTD, Basingstoke, Hampshire, England) with 5% sheep blood in a 5% CO_2 atmosphere. The *O. rhinotracheale* bacteria were transferred into brain heart infusion (BHI) broth (Oxoid) for 24 h at 37°C with agitation. The bacterial challenge inoculum was prepared by washing the cultured bacteria twice in phosphate-buffered saline (PBS) followed each time by five minutes of centrifugation at 3,000 rpm at 4°C. The resulting pellet was resuspended in PBS to obtain a final concentration of 10^8 colony-forming units (cfu)/ml. Confirmation of the number of cfu/ml was done by inoculating ten-fold dilutions in PBS on sheep blood agar and counting the number of colonies.

Experimental Design

In a first trial (dose titration study), 85 turkeys were randomly divided into 5 groups and inoculated with $10^{4.5}$ CD_{50} of APV at 25 days of age. All groups, except group NC, were inoculated with $10^{8.5}$ cfu of *O. rhinotracheale*, 3 days later. Group NC was enclosed as an untreated negative control group. At 24 h after *O. rhinotracheale* inoculation, 3 groups received antimicrobial treatment by medicating the drinking water during 5 successive days. The first group received enrofloxacin at 5 mg/kg (group E5-5D), the second group at 10 mg/kg (group E10-5D) and the third group at 20 mg/kg (group E20-5D).

In a second trial (reduced duration medication study) 68 turkeys were randomly divided into 4 groups of 17 animals. At 20 days of age the birds of all groups were inoculated with $10^{4.4}$ CD_{50} of APV and 3 days later with $10^{8.8}$ cfu of *O. rhinotracheale*. Three groups received enrofloxacin treatment by medicating the drinking water starting at 24 h after *O. rhinotracheale* inoculation. Group E10-5D was treated at a dose of 10 mg / kg BW daily for 5 successive days. Group E25-2D was treated at a dose of 25 mg / kg BW / day on day 0 and day 2 and group E15-3D at a dose of 15 mg / kg BW / day on days 0, 2 and 4.

In both trials group PC was included as an untreated positive control group. Viral and bacterial inoculation occurred by dividing a total of 250 µl of inoculum equally over the nostrils and eyes.

In every group, water uptake was measured on a daily basis from three days before until the end of the treatment periods. Additionally, all animals were weighed before *O. rhinotracheale* inoculation. On the basis of these data, the antimicrobial concentrations to be administered in the water and the actually received dosages were calculated. Water samples from the medicated drinking water were collected daily to determine the actual amount of enrofloxacin using turbulent flow chromatography/tandem mass spectrometry (Krebber, 2003).

All birds were examined for clinical signs on a daily basis throughout the experiment until they were sacrificed. The clinical signs of the upper respiratory tract (nasal exudates, swollen sinuses, frothy eyes) were scored as described in Van de Zande *et al*. (2001). The clinical condition of each bird was assigned a score from 0 (absence of clinical signs) to 6 (death).

Tracheal swabs were taken from all birds two days post viral inoculation (pvi) for APV enumeration and at regular time points post bacterial inoculation (pbi) for titration of *O. rhinotracheale.* The tracheal swabs were collected using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc.**,** Corona, USA) and placed in transport medium. The latter consisted of 1 ml PBS for bacteriological examination which was supplemented with 10%

fetal calf serum (Gibco, Invitrogen Corporation, Merelbeke, Belgium), penicillin (1000 U/ml) (Biopharma, Rome, Italy) and kanamycin (0.5 mg/ml) (Gibco) for virus enumeration.

Five birds of each group were sacrificed at 6 days pbi. The birds were necropsied and examined for the presence of gross lesions. Samples of the turbinates, trachea and lungs were collected and a 10% tissue suspension in PBS was prepared for *O. rhinotracheale* examination. Air sacs, pericardium and liver were sampled with cotton swabs for *O. rhinotracheale* re-isolation.

Equal numbers of the remaining birds of each group were necropsied and examined for the presence of gross lesions at 15 and 16 days pbi (trial 1) or 14 and 15 days pbi (trial 2). Turbinates, trachea, lungs, air sacs, liver and pericardium were sampled with cotton swabs for *O. rhinotracheale* re-isolation.

All samples were processed immediately after collection as described below.

The birds were weighed the day before the APV inoculation, the day before the *O. rhinotracheale* inoculation and subsequently the final day of the experiment.

These experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Virological Enumeration and Bacteriological Titration of Tracheal Swabs

The CD_{50} per ml of APV and the cfu per ml of *O. rhinotracheale* were determined, using procedures described in Marien *et al.* (2005). In addition, in order to detect *O. rhinotracheale* isolates with reduced susceptibility to enrofloxacin, samples were also inoculated on sheep blood agar supplemented with 5 µg/ml gentamicin, 5 µg/ml polymyxin and 0.25 µg or 2 µg enrofloxacin/ml.

Bacteriological Examination of Tissue Suspensions and Swabs

From samples of the turbinates, trachea, and lungs, the cfu of *O. rhinotracheale* was determined in duplicate by incubating undiluted tissue suspensions and tenfold serial dilutions of tissue suspensions on Columbia Blood Agar supplemented with 5 µg/ml gentamicin and 5 µg/ml polymyxin. After 24-48 h of incubation at 37°C in a 5% CO_2 atmosphere, viable counts were performed and the number of cfu/ml tissue suspension calculated.

The swabs taken from the air sacs, pericardium and liver from the 25 birds sacrificed at 6 days pbi and the swabs from the turbinates, trachea, lungs, air sacs, pericardium and liver from the birds sacrificed at 15 and 16 days pbi (trial 1) or at 14 and 15 days pbi (trial 2) were inoculated on Columbia Blood Agar supplemented with 5 µg/ml gentamicin and 5 µg/ml

polymyxin. After 24-48 h of incubation at 37°C in a 5% CO_2 atmosphere, the agar was examined for the presence of *O. rhinotracheale* colonies.

Statistical Analyses

Tracheal titres of *O. rhinotracheale* were analysed for all birds using the measurements between 0 and 6 days pbi and subsequently from the birds that were not euthanized between 0 and 9 days pbi. The clinical scores and weight were analysed only for animals that were not euthanized at 6 days pbi.

The average over time for the clinical scoring and tracheal swabbing was analysed using a fixed-effects model with the treatments group as fixed effect.

Weight gain was compared at the end of the trial using a fixed-effects model.

O. rhinotracheale titres from the turbinates, trachea and lung of the birds, euthanized at 6 days pbi were compared between the five treatment groups with the Kruskall-Wallis test, whereas the presence of *O. rhinotracheale* in the airsacs, liver and pericardium of the animals, euthanized at 6 days pbi and the presence of *O. rhinotracheale* in the turbinates, trachea, lungs, airsacs, liver and pericardium of the birds euthanized 15 and 16 days pbi, were compared between the five treatments with the Fisher's exact test. For the presence of *O. rhinotracheale* in the turbinates at final necropsy also the Chi-Square test was used.

All tests were performed at a global 5% significance level and the pairwise comparisons were tested at a significance level adjusted using Bonferroni's multiple comparisons technique (Agresti, 2002).

Results

Antimicrobial Uptake

Trial 1. Dose Titration Study. The mean actual daily dose for the total medication period was 5.36 mg/kg (range 3.9-6.1) for group E5-5D, 10.16 mg/kg (range 9.6-11.7) for group E10-5D mg/kg and 20.8 mg/kg (range 18.1-25.8) for group E20-5D.

Trial 2. Reduced Duration Medication Study. The mean actual daily dose for the total medication period was 9.8 mg/kg (range 9.0-10.5) for group E10-5D, 15.5 mg/kg (range 15.0-16.0) for group E15-3D mg/kg and 24.7 (range 22.0-27.4) for group E25-2D.

Clinical Signs

Trial 1. Dose Titration Study. No animals died due to the APV/*O. rhinotracheale* infections. The mean clinical scores calculated for each group are presented in Figure 1.

Compared to the positive control group, clinical signs were significantly reduced as well as severity and duration in every enrofloxacin treatment group. The negative control group, only inoculated with APV, had lower clinical scores compared to all other infected groups at all time points.

Data presented in Table 1 show the area under the curve (AUC) scores from 0 to 9 days pbi. The treated groups had significantly lower AUC scores than the positive control ($P \leq 0.037$). When the three enrofloxacin treatments were compared with each other, no significant difference was noted ($P \geq 0.349$). All treatments differed significantly from the negative control which had higher clinical scores ($P \leq 0.024$).

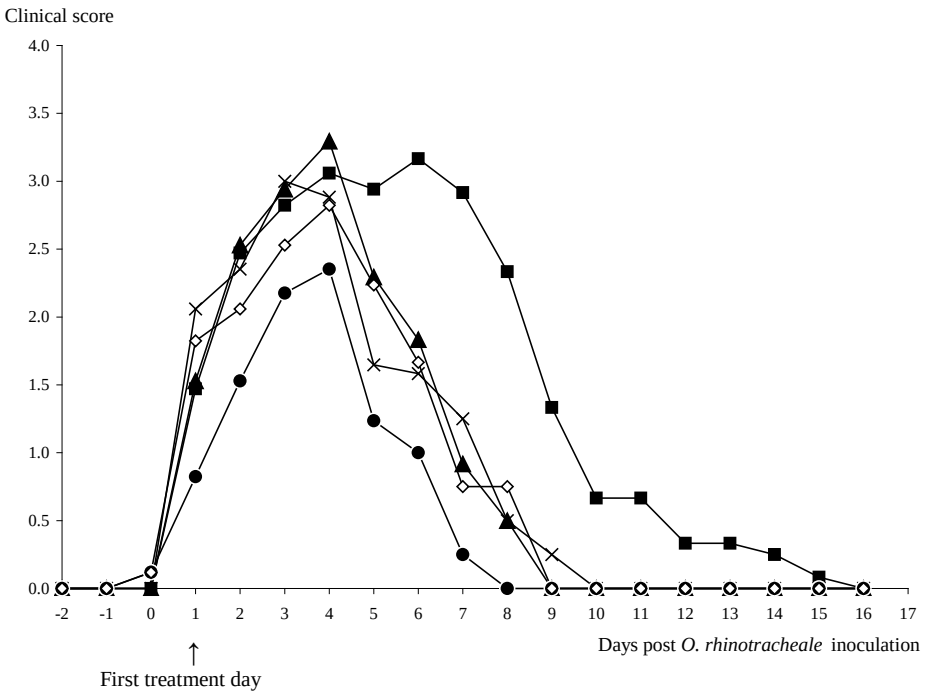

Figure 1. Mean clinical scores in turkeys, inoculated with APV followed by *O. rhinotracheale* three days later, and treated for 5 successive days with 5 mg/kg/day enrofloxacin (group E5-5D ▲), 10 mg/kg/day enrofloxacin (group E10-5D ◊) or 20 mg/kg/day enrofloxacin (group E20-5D X). The positive control group (group PC ■), infected with APV and *O. rhinotracheale*, and the negative control group (group NC ●), infected with APV remained untreated.

Trial 2. Reduced Duration Medication Study. One turkey died in group E25-2D four days after the final treatment day, but according to necropsy and bacteriological investigation,

this was due to an *Enterococcus faecalis* infection. As no relation to bacterial inoculation or study medication was encountered, this turkey was excluded from all statistical analyses.

The mean clinical scores calculated for each group are presented in Figure 2.

Compared to the positive control group clinical signs in the treated groups were less pronounced (group E10-5D and E25-2D) or the higher scores reduced over time (group E15-3D). In addition, clinical signs in the treated groups disappeared two days (group E15-3D and group E10-5D) or one day (group E25-2D) earlier, than the clinical scores in the positive control group.

From 7 days pbi onwards, one bird from group E25-2D suffered from an airsac rupture. Since no bacterial organism could be isolated out of the airsacs of this turkey during final necropsy, this was probably due to trauma.

Data presented in Table 2 show the AUC scores from 0 to 9 days pbi. Comparisons between pairs of *O. rhinotracheale* infected groups revealed significant differences between group E10-5D and the positive control group (P = 0.0085) and also between group E15-3D and the positive control group (P = 0.0244).

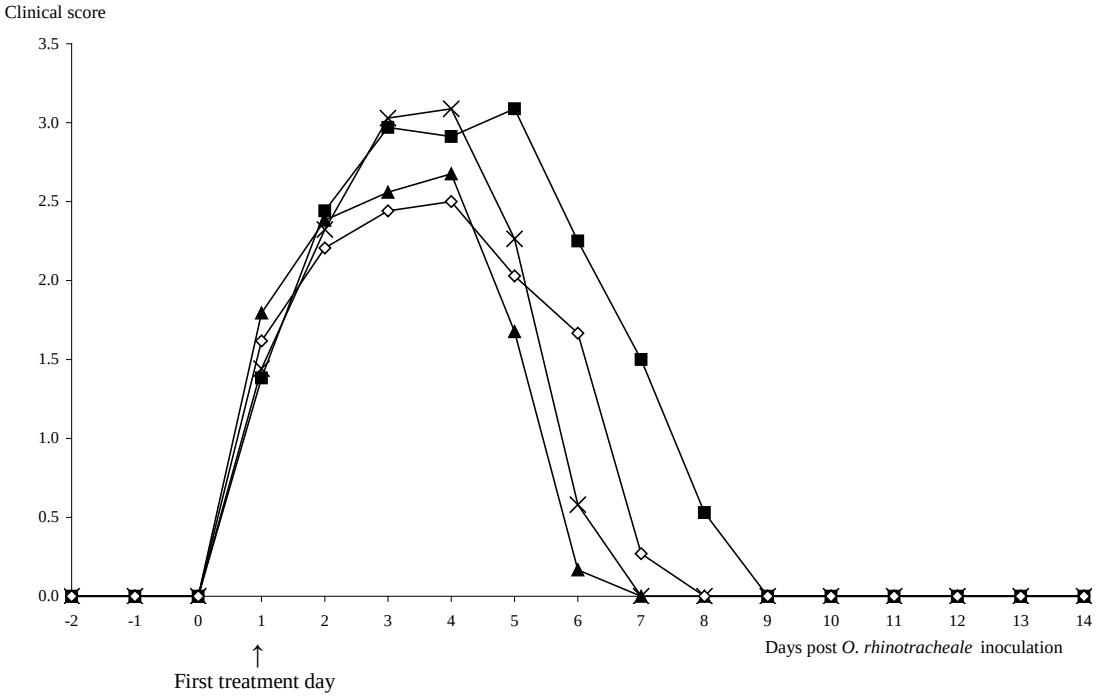

Figure 2. Mean clinical scores in turkeys, inoculated with APV followed by *O. rhinotracheale* three days later, and treated with 10 mg/kg enrofloxacin for 5 days (group E10-5D ▲), with 25 mg/kg enrofloxacin for 2 days alternated with a non treating day (group E25-2D ◊) and with 15 mg/kg enrofloxacin for 3 days alternated each time by a non treating day (group E15-3D X). The positive control group (group PC ■) remained untreated.

Table 1. Observations of gross lesions at 6 days post bacterial infection and at 15 or at 16 days post bacterial infection of turkeys inoculated with APV followed by *O. rhinotracheale* three days later and treated with different enrofloxacin regimens: 5 mg/kg for 5 successive days (E5-5D), 10 mg/kg for 5 successive days (E10-5D) and 20 mg/kg for 5 successive days (E20-5D). The positive control group (PC), infected with APV and *O. rhinotracheale*, and the negative control group (NC), infected with APV remained untreated.

Necropsy days pbi	Lesion type	NC	PC	E5-5D	E10-5D	E20-5D
6	rhinitis (nostrils, conchae)	1/5 (*)	5/5	5/5	3/5	1/5
	sinusitis	3/5	5/5	2/5	4/5	3/5
	tracheitis	0/5	0/5	1/5	0/5	0/5
	pneumonia	0/5	0/5	0/5	0/5	0/5
	airsacculitis	0/5	2/5	0/5	0/5	0/5
	All lesions	4/25 (16%)	12/25 (48%)	8/25 (32%)	7/25 (28%)	4/25 (16%)
15 or 16	rhinitis (nostrils,conchae)	0/12	3/12	1/12	0/12	0/12
	sinusitis	0/12	2/12	0/12	0/12	0/12
	tracheitis	0/12	0/12	0/12	0/12	0/12
	pneumonia	0/12	0/12	0/12	0/12	0/12
	airsacculitis	0/12	1/12	0/12	0/12	0/12
	All lesions	0/60 (0%)	6/60 (10%)	1/60 (2%)	0/60 (0%)	0/60 (0%)

(*) number of positive observations/total number of observations

Gross Findings and Lesion Observations

Trial 1. Dose Titration Study. The observations of gross lesions in the organs of the birds necropsied at 6 days pbi and 15 or 16 days pbi are shown in Table 1. Although no significant statistical differences between the different groups were noted, the highest percentage (48%) of clinically affected organs was seen in the positive control group. This group also showed the most severe lesions. Each turkey in the positive control group showed mucoserous exudate in the upper respiratory tract (nostrils, turbinates and sinuses) and two birds also suffered from airsacculitis. In the other groups, lesions were limited to the upper respiratory tract. When the three enrofloxacin treatment groups were compared, fewer birds with lesions were seen in group E20-5D (16%) than in group E5-5D (32%) and group E10-5D (28%). These latter groups also showed more mucous exudate in the upper respiratory tract while the exudate found in birds from group E20-5D was always clear. Comparing E20-5D with the negative control group (which was only infected with APV) equal percentages of organs affected were found (16%) and gross lesions were relatively similar.

During final necropsy at 15 and 16 days pbi, three birds from the positive control group and one bird from group E5-5D suffered from serous exudate in the upper respiratory tract. One bird from the positive control group also showed condensed airsacs. No statistically significant difference between the groups could be noted.

Trial 2. Reduced Duration Medication Study. The observations of gross lesions in the organs of the birds necropsied at 6 days pbi and 14 or 15 days pbi are shown in Table 2.

Most severe lesions and highest percentage of affected organs (60%) were seen in the positive control group. Each turkey in this group showed mucous exudate in the upper respiratory tract (nostrils, turbinates and sinuses). Four birds also suffered from pneumonia and one from airsacculitis. In the treated turkey groups, not every bird was affected. One turkey from group E15-3D and 10E-5D and two turkeys from E25-2D did not show any gross lesions. Lesions in the affected birds (serous to mucous exudate) were limited to the upper respiratory tract, except for one bird from group E10-5D which showed hyperaemic air sacs. Observations of gross lesions in the three enrofloxacin treated groups were highly similar.

Only a statistical difference was noticed for pneumonia ($P = 0.0026$ related to the chi-square test). Pairwise comparisons with the Fisher's exact test for each pair of experimental groups resulted in significant differences ($P = 0.0476$) for each comparison of an enrofloxacin treatment group to the positive control group.

Table 2. Observations of gross lesions at 6 days post bacterial infection and at 14 or at 15 days post bacterial infection of turkeys inoculated with APV followed by *O. rhinotracheale* three days later and treated with different enrofloxacin regimens: 10 mg/kg for 5 successive days (E10-5D), 25 mg/kg enrofloxacin for 2 days alternated with a non treating day (E25-2D) and 15 mg/kg enrofloxacin for 3 days alternated each time by a non treating day (E15-3D). The positive control group (PC) remained untreated.

Necropsy days pbi	Lesion type	PC	E15-3D	E25-2D	E10-5D
6	rhinitis (nostrils, conchae)	5/5(*)	1/5	3/5	3/5
	sinusitis	5/5	4/5	2/5	3/5
	tracheitis	0/5	0/5	0/5	0/5
	pneumonia	4/5	0/5	0/5	0/5
	airsacculitis	1/5	0/5	0/5	1/5
	All lesions	15/25 (60%)	5/25 (20%)	5/25 (20%)	7/25 (28%)
14 or 15	rhinitis (nostrils, conchae)	0/12	1/12	0/11	0/12
	sinusitis	0/12	0/12	0/11	0/12
	tracheitis	0/12	0/12	0/11	0/12
	pneumonia	0/12	0/12	0/11	0/12
	airsacculitis	2/12	0/12	1/11	0/12
	All lesions	2/60 (3%)	1/60 (2%)	1/55 (2%)	0/60 (0%)

(*) number of positive observations/total number of observations

During final necropsy at 14 and 15 days pbi, several birds in every turkey group showed congested lungs or an injected pericardium, a side effect of T61 euthanasia. No statistically significant results regarding the observations of lesions at the final necropsies could be observed between the different turkey groups.

Virological Titration from Tracheal Swabs

Virological titration of tracheal swabs collected at two days post viral infection, showed that APV was recovered from every bird.

Trial 1. Dose Titration Study. Mean titres were $10^{4.5}$ CD_{50}/ml, $10^{4.5}$ CD_{50}/ml, $10^{4.3}$ CD_{50}/ml, $10^{4.5}$ CD_{50}/ml and $10^{4.3}$ CD_{50}/ml for group E5-5D, group E10-5D, group E20-5D, negative control group and the positive control group respectively.

Trial 2. Reduced Duration Medication Study. Mean titres were $10^{4.3}$ CD_{50}/ml, $10^{4.5}$ CD_{50}/ml, $10^{4.3}$ CD_{50}/ml and $10^{4.3}$ CD_{50}/ml for group E10-5D, group E15-3D, group E25-2D and the positive control group respectively.

Bacteriological Titration of Tracheal Swabs

Trial 1. Dose Titration Study. The results of the *O. rhinotracheale* counts of the tracheal swabs are shown in Figure 3.

O. rhinotracheale was not isolated at any time during the experiment in the negative control group, which was only infected with APV.

In contrast with the non-treated positive control group, *O. rhinotracheale* was never isolated out of any trachea in any enrofloxacin treatment group after the first treatment day, with the exception of group E10-5D, in which *O. rhinotracheale* was re-isolated occasionally out of the trachea of two birds, starting from five days after the last treatment day (10 days pbi), but only at low numbers and without any marked clinical symptoms.

Using the AUC in the fixed-effects model, all enrofloxacin treatments differed significantly from the positive control with lower *O. rhinotracheale* counts ($P < 0.001$). There was no significant difference between the enrofloxacin treatment regimens ($P \geq 0.18$) (Table 3).

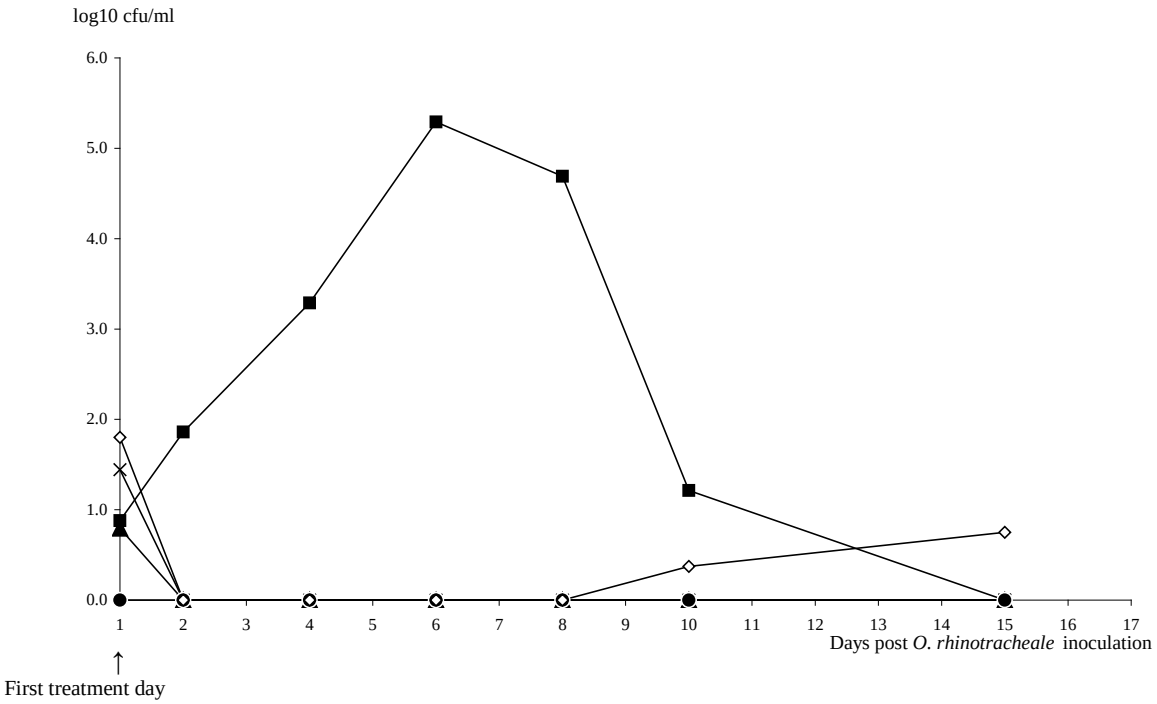

Figure 3. Mean cfu/ml of *O. rhinotracheale* in tracheal swabs collected from turkeys, inoculated with APV followed by *O. rhinotracheale* three days later and treated for 5 successive days with 5 mg/kg/day enrofloxacin (group E5-5D ▲), 10 mg/kg/day enrofloxacin (group E10 -5D ◊) or 20 mg/kg/day enrofloxacin (group E20-5D X). The positive control group (group PC ■), infected with APV and *O. rhinotracheale*, and the negative control group (group NC ●), infected with APV remained untreated.

Trial 2. Reduced Duration Medication Study. The results of the *O. rhinotracheale* counts of the tracheal swabs are shown in Figure 4.

In contrast with the positive control group, *O. rhinotracheale* was never isolated out of a trachea in any enrofloxacin treatment group after the first treatment day in group E10-5D and group E25-2D or after the second treatment day in group E15-3D.

Using the AUC in a fixed effects-model, all enrofloxacin treatment regimens differed significantly from the positive control group ($P < 0.0001$). There was no significant difference between the enrofloxacin treatment regimens ($P > 0.05$) (Table 4).

Tracheal swabs were plated on enrofloxacin supplemented agar plates (i.e. 0.25 µg/ml agar and 2 µg/ml agar) and did not show any growth of less susceptible or resistant *O. rhinotracheale* isolates at any time during both experiments.

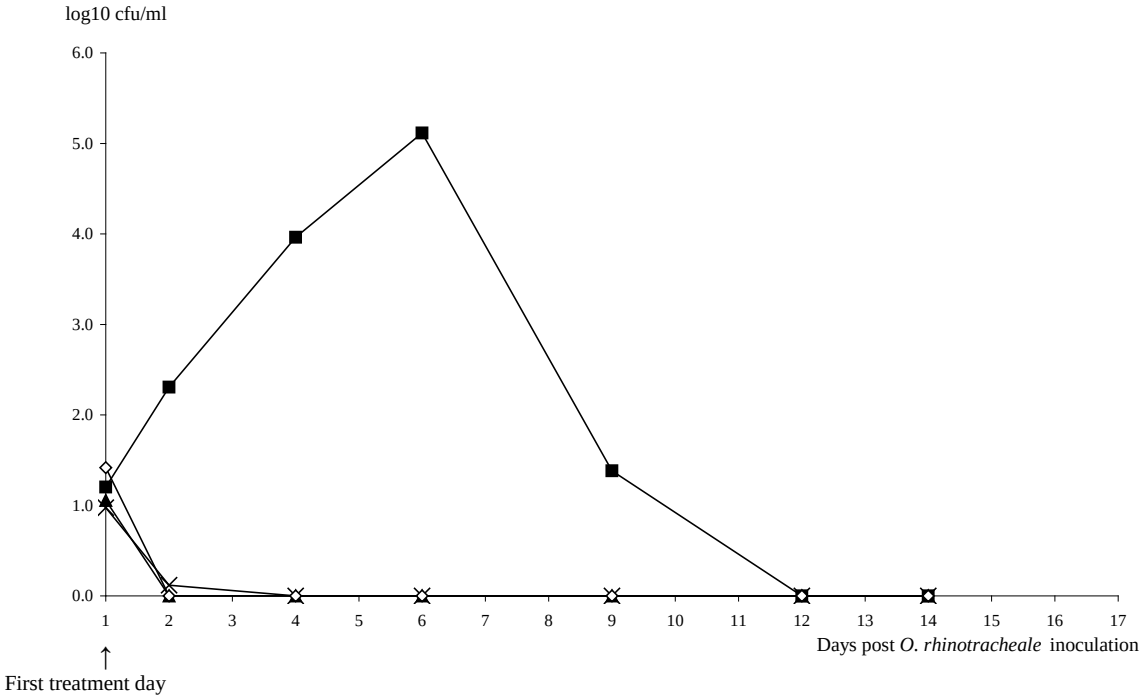

Figure 4. Mean cfu/ml of *O. rhinotracheale* in tracheal swabs collected from turkeys, inoculated with APV followed by *O. rhinotracheale* three days later and treated with 10 mg/kg enrofloxacin for 5 days (group E10-5D ▲), with 25 mg/kg enrofloxacin for 2 days alternated with a non treating day (group E25-2D ◊) and 15 mg/kg enrofloxacin for 3 days alternated each time by a non treating day (group E15-3D X). The positive control group (group PC ■) remained untreated.

Bacteriological Examination of Tissue Suspensions and Swabs

Trial 1. Dose Titration Study. The mean number of cfu/ml of *O. rhinotracheale* in the organs of the birds necropsied at 6 days pbi are shown in Table 3.

During necropsy at 6 days pbi, *O. rhinotracheale* was only isolated from the respiratory organs (turbinates, trachea, lungs and airsacs) of the positive control group.

Statistically significant differences were observed for comparison between the enrofloxacin treatments and the positive control with lower *O. rhinotracheale* cfu counts (for air sacs, $P < 0.035$; for lungs, $P < 0.001$; for tracheas, $P < 0.001$ and for turbinates $P < 0.001$) in the treated groups. There was no statistically significant difference between the enrofloxacin treatment regimens.

Table 3. Clinical scores and isolation of *O. rhinotracheale* out of tracheal swabs and organs (mean value ± standard error) of turkeys inoculated with APV followed by *O. rhinotracheale* three days later, and treated with different enrofloxacin regimens: 5 mg/kg for 5 successive days (E5-5D), 10 mg/kg for 5 successive days (E10-5D) and 20 mg/kg for 5 successive days (E20-5D). The positive control group (PC), infected with APV and *O. rhinotracheale*, and the negative control group (NC), infected with APV remained untreated.

	Clinical Scores *	Isolation of *O. rhinotracheale* out of tracheal swabs**	Isolation of *O. rhinotracheale* at 6 days pbi (log_{10}cfu/g tissue)		
Group	Mean AUC	Mean AUC	Turbinates	Trachea	Lungs
NC	9.3 ± 5.3 [c]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.00 ± 0.0 [b]	0.00 ± 0.0 [b]
PC	22.3 ± 6.0 [a]	25.0 ± 4.4 [a]	6.8 ± 0.5 [a]	6.6 ± 0.5 [a]	4.3 ± 0.3 [a]
E5-5D	16.1 ± 6.9 [b]	0.6 ± 0.5 [b]	0.0 ± 0.0 [b]	0.00 ± 0.0 [b]	0.00 ± 0.0 [b]
E10-5D	15.0 ± 4.6 [b]	0.9 ± 0.5 [b]	0.0 ± 0.0 [b]	0.00 ± 0.0 [b]	0.00 ± 0.0 [b]
E20-5D	17.1 ± 6.8 [b]	0.7 ± 0.6 [b]	0.0 ± 0.0 [b]	0.00 ± 0.0 [b]	0.00 ± 0.0 [b]

[a] treatments with different superscripts differ significantly from each other at the 5% global significance level
* from 0 to 9 days post bacterial inoculation (days pbi), ** from 0 to 8 days pbi

Table 4. Clinical scores and isolation of *O. rhinotracheale* out of tracheal swabs and organs (mean value ± standard error) of turkeys inoculated with APV followed by *O. rhinotracheale* three days later, and treated with different enrofloxacin regimens: 10 mg/kg for 5 successive days (E10-5D), 25 mg/kg enrofloxacin for 2 days alternated with a non treating day (E25-2D) and 15 mg/kg enrofloxacin for 3 days alternated each time by a non treating day (E15-3D). The positive control group (PC) remained untreated.

	Clinical Scores *	Isolation of *O. rhinotracheale* out of tracheal swabs*	Isolation of *O. rhinotracheale* at 6 days pbi (log_{10}cfu/g tissue)		
Group	Mean AUC	Mean AUC	Turbinates	Trachea	Lungs
PC	13.4 ± 5.4 [a]	27.6 ± 5.3 [a]	6.6 ± 1.1 [a]	6.7 ± 0.4 [a]	4.0 ± 0.2 [a]
E15-3D	9.8 ± 3.1 [b]	0.7 ± 1.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]
E25-2D	11.1 ± 3.3 [ab]	0.6 ± 0.6 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]
E10-5D	9.1 ± 3.1 [b]	0.6 ± 0.4 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]

[a] treatments with different superscripts differ significantly from each other at the 5% global significance level
* from 0 to 9 days post bacterial inoculation (days pbi)

During necropsy days 15 and 16 days pbi, one turkey belonging to group 10E-5D, showed presence of *O. rhinotracheale* in the turbinates and two turkeys from the same groups in the turbinates and in the trachea. These were the same birds in which *O. rhinotracheale* was re-isolated from the tracheal swabs, five days after the final treatment day. *O. rhinotracheale* was not isolated from any other organ in any other bird. In the trachea, the difference between treatments was not statistically significant ($P \geq 0.1$). In the turbinates, the Chi-Square test concluded in significant differences between treatments ($P = 0.022$) while the Fisher exact test was statistically borderline ($P = 0.051$).

Trial 2. Reduced Duration Medication Study. The mean number of cfu/ml of *O. rhinotracheale* in the organs at 6 days pbi are shown in Table 4.

During first necropsy 6 days pbi, *O. rhinotracheale* was also only isolated out of the respiratory organs (turbinates, trachea, lungs and airsacs) from the positive control group.

Statistically significant differences regarding the *O. rhinotracheale* titres in the turbinates, trachea and lungs were observed for all comparisons of the three enrofloxacin treatment groups to the positive control group (for every comparison $P = 0.0079$). For these organs (turbinates, trachea and lungs), no statistical differences were observed between the three enrofloxacin treatment regimens. Also no statistical differences were observed between the four experimental groups regarding the presence of *O. rhinotracheale* in the airsacs, pericard and liver.

During final necropsy, *O. rhinotracheale* could not be isolated out of any organ.

Weight

During both trials, no global treatment effect on weight gain could be observed. In trial 1 the positive control group showed the lowest weight gain and there was one single significant pairwise difference for the 81 g higher weight gain observed in E10-5D compared to the positive control group ($P = 0.0495$). In trial 2 group E15-3D showed the highest weight gain. However, again this difference was not statistically significant

Discussion

The infection model used in these experiments was based on a model developed by Marien *et al.* (2005) for the reproduction of infectious respiratory disease in turkeys. Since the results obtained in the control groups in both experiments are very similar to their findings, the infection model appears to be highly reproducible.

Regarding the clinical symptoms in the dose titration study (trial 1), the negative control group scored statistically significant better than the other groups. This was expected since the negative control group was only infected with APV. It is generally acknowledged that *O. rhinotracheale* exerts a synergistic effect on APV infections in turkeys by aggravating the symptoms encountered in the respiratory tract (Van Empel *et al.,* 1996; Jirjis *et al.,* 2004, Marien *et al.,* 2005). Looking at the clinical scores and the respiratory lesions observed during necropsy in the positive and negative control group, this experiment (trial 1) confirms these findings.

Taking in consideration that, compared to the negative control group, the course of clinical disease in the enrofloxacin treated groups in the dose titration study (trial 1) was only prolonged for a day and that no significant differences could be noted for the gross lesions encountered during necropsy, it can be suggested that the viral multiplication of APV had a major impact on the overt respiratory symptoms in the three enrofloxacin treated groups. In the APV/ *O. rhinotracheale* infected, untreated birds, however, clinical disease was more severe. Thus, the multiple day enrofloxacin treatments were able to eliminate most of the *O. rhinotracheale* associated aggravation of the APV infection.

Statistical analysis confirms these findings as each enrofloxacin treatment regimen, used in trial 1, showed statistically significant better results for most parameters examined, compared to the APV/*O. rhinotracheale* infected, untreated positive control group (i.e. clinical scoring, *O. rhinotracheale* titres from tracheal swabs and *O. rhinotracheale* titres from organs 6 days pbi). Comparable results were observed in the reduced duration medication study (trial 2). Here also with every enrofloxacin treatment statistically significant better results were obtained regarding clinical score (with the exception of the group treated with 25 mg/kg for two days, although capable of limiting the clinical symptoms and shortening the course of respiratory disease), the *O. rhinotracheale* titres in the tracheas, the *O. rhinotracheale* counts in the organs and the observations of pneumonia at necropsy 6 days pbi, compared to the positive control group. As no adverse reactions were observed the reduced duration medication was apparently well tolerated. However the alternative medication regimens with higher daily doses at fewer medication days could affect the drug depletion period and subsequent withdrawal time.

Both in the dose titration study and in the reduced duration medication study no statistical differences could be noted between the different enrofloxacin treatments. Every enrofloxacin regimen resulted, in both trials, in an equal reduction of clinical signs and equal shortening of the course of respiratory disease. Moreover, all regimens were able to eliminate the *O.*

rhinotracheale infection from the diseased turkeys since no *O. rhinotracheale* bacteria could be isolated out of any trachea or organ after the first treatment day. Such clearance of *O. rhinotracheale* could not be obtained after the administration of a single day dose of 50 mg/kg which resulted in the re-emergence of the bacterium after treatment (Garmyn *et al*., 2009). Re-emergence was not observed when the 50 mg/kg dose was split over a two or three day period.

Overall, for the treatment of *O. rhinotracheale* infections with an enrofloxacin sensitive strain, a five day treatment regimen of 20 mg enrofloxacin/kg was not shown to be more efficacious than the labelled dosage (10 mg enrofloxacin/kg daily for 5 days) and a five day treatment regimen of 5 mg enrofloxacin/kg was found equally efficient as the labelled dosage. Whether under field circumstances, with frequent *O. rhinotracheale/ E. coli* mixed infections involved in turkey respiratory disease, clinical disease could be controlled with the 5 mg/kg regimen remains to be determined. The labelled dosage for enrofloxacin for respiratory colibacillosis is 10 mg/kg BW. Also reduced duration medication could be a valuable alternative to the labelled dosage against *O. rhinotracheale* infections in turkeys, providing that the total administered dose (50 mg/kg) is complied with i.e. 2 times 25 mg/kg or 3 times 15 mg/kg, alternated each time by a day without treatment. This would improve user convenience by reducing the workload for the farmer who will only have to prepare medicated water for two or three days instead of five. In a previous study performed by Marien *et al.* (2006) an enrofloxacin drinking water treatment of 3 or 5 continuous days showed to be equally effective. The results obtained in this study suggest that every other day treatment with enrofloxacin would seem to be of value. Hence, it is demonstrated that when enrofloxacin is used in the drinking water, a flexible adaptation of the duration of treatment, depending on the evolution of the respiratory disease in the stable, is possible.

Five days after the final enrofloxacin administration in the dose titration study (trial 1), low titres of *O. rhinotracheale* were isolated out of two birds from the group receiving 10 mg enrofloxacin/kg daily for 5 days. This tracheal re-colonization, several days after the final treatment day, was not seen in the lower and higher dose group, and is expected not to be dose dependent. It is reported that *O. rhinotracheale* can survive for several days in the environment (Lopes *et al.*, 2002). Therefore this re-excretion or re-infection, which was not accompanied by clinical relapse, is likely just incidental.

Acquired resistance to enrofloxacin is commonly encountered in *O. rhinotracheale* (Devriese *et al*., 1995; Malik *et al.*, 2003) and is the consequence of stepwise mutations in the *gyr* and *par* genes (Barnard and Maxwell, 2001; Hooper, 2001). In these studies, however, *O.*

rhinotracheale clones with reduced susceptibility or resistance were not detected in any of the enrofloxacin treatment groups.

In conclusion, the enrofloxacin treatments tested in the dose titration study (5, 10 or 20 mg enrofloxacin/kg, daily for 5 days) and the reduced duration medication study (2 x 25 mg enrofloxacin/kg, 3 x 15 mg enrofloxacin/kg, alternated each time by a day without treatment and 10 mg enrofloxacin/kg, daily for 5 days) were equally capable of shortening the course of clinical disease, eliminating *O. rhinotracheale* from the respiratory tract and reducing gross lesions. Since little differences could be observed for the clinical scores and the gross lesions, when the enrofloxacin treatment groups and the negative control group were compared, the viral multiplication of APV had a major impact on the overt respiratory disease symptoms in all experimental groups. However, all multiple day enrofloxacin treatments were able to eliminate most of the *O. rhinotracheale* associated aggravation of the APV infection, which resulted in more severe and long-lasting respiratory symptoms in the non treated positive control group.

As no *O. rhinotracheale* bacteria grew on the enrofloxacin supplemented media, none of the used treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance.

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CHAPTER 4: GENERAL DISCUSSION

GENERAL DISCUSSION

Antimicrobial use in poultry production: a hot and controversial topic

Antimicrobial use in the poultry industry is currently under scrutiny. Emerging antimicrobial resistance is becoming an increasing problem in most bacterial species. For *O. rhinotracheale* reduced susceptibility or resistance have been reported against amoxicillin, ampicillin, doxycycline, enrofloxacin, flumequine, gentamicin, lincomycin, trimethoprim-sulfonamide, tetracycline and tylosin (Van Empel and Hafez, 1999; Devriese *et al.*, 2001; van Veen *et al.*, 2001; Malik *et al.*, 2003; Soriano *et al.*, 2003). Avian pathogenic *E. coli* are frequently (multi-)resistant to tetracyclines, sulfonamides, ampicillin, and streptomycin (Barnes *et al.*, 2008), but also for other antimicrobials commonly used in the poultry industry reports of reduced susceptibility or resistance exist (Cormican *et al.*, 2001; Altekruse *et al.*, 2002; Barnes *et al.*, 2008). For both bacterial species susceptibility may depend on the regime used by the poultry industry in various geographical locations (Barnes *et al.*, 2008).

Concerns are also growing about antimicrobial resistance in bacteria from food animals, and their impact on human health. These resistant strains may be zoonotic, but also resistant commensals pose a hazard, since they can transfer their resistance genes to human pathogens. Heated discussions regarding the degree of risk this hazard poses, have been and are still being held between those who are very concerned about the risk (Witte, 1998) and those who believe that the evidence for a substantial risk remains sparse (Phillips *et al.*, 2004).

Many risk assessments have been conducted, but in many cases the results were impended by shortage of suitable data sets (Snary *et al.*, 2004). In addition, a number of countries have incorporated surveillance programs, e.g. National Antimicrobial Resistance Monitoring System (NARMS), European Antimicrobial Susceptibility Surveillance in Animals (EASSA) or Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP), but these monitoring programs do not yet provide adequate information regarding the risk of antimicrobial resistance, which is therapeutic failure due to a specific drug resistance in humans (Mathew, 2007). Although no definite conclusion has come from the several meetings designed to discuss the question of antimicrobial resistance and the use of antibiotics in poultry, the controversy has led to legislative changes affecting the availability of antimicrobials approved for use in poultry. From September 2005, the US Food and Drug Administration banned the use of enrofloxacin in poultry farms in the USA (not in Europe) as it was expected to jeopardize human health through the emergence of

fluoroquinolone-resistant strains of *Campylobacter* in enrofloxacin medicated poultry (FDA, 2005). Since January 2006, the water -or feed- based administration of antimicrobials to animals as growth promoters has been completely banned in the European Community as such use has been shown to contribute to the increased prevalence of antimicrobial-resistant bacteria of human significance (e.g. enterococci). It is certainly true that the widespread practice of mass dosing antimicrobials in the drinking water, using sub-therapeutic levels promotes the emergence of resistance (Iovine and Blaser, 2005; Johnsen *et al.*, 2009). It is also true that removal of the selective pressure by abolishing the use of a certain antimicrobial can reduce the frequency of resistance (Han *et al.*, 2009; Johnsen *et al.*, 2009). However, complete reversal of acquired drug resistance in bacterial populations that were no longer exposed to a specific antimicrobial drug has never been reported (Sepala *et al.*, 1997; Enne *et al.*, 2001; Sorum *et al.*, 2006; Price *et al.*, 2007) and any use of antimicrobial drug may eventually lead to resistance. Redundant use of antimicrobials should certainly be avoided and the use of antimicrobials should certainly not be a crowning piece of disease control programmes. Yet, a ban of the use of an antimicrobial product has implications for animal welfare by limiting efficacious drugs to treat disease. Therapeutic applications of antimicrobials are vital for profitable and livestock production, essential for animal welfare and minimizing economic losses (McMullin, 2004). To retain the efficacy of antimicrobial drugs currently available for the control of bacterial infections, prescription and administration of antimicrobial agents should be undertaken with discretion, supported by an accurate diagnosis and a careful choice of the respective substance(s) (Schwarz *et al.*, 2001).

Efficacy of the labelled enrofloxacin regimen

The principal goal of therapeutic antimicrobial use in poultry is maximum eradication of the pathogen as quickly as possible with minimal adverse effects on the recipient and prevention of the emergence of resistant organisms (Tanner *et al.*, 2003).

Currently, enrofloxacin is the most potent antimicrobial drug so far used for respiratory infections in poultry medicine. From all fluoroquinolone antimicrobials which are approved for poultry use, enrofloxacin shows the highest *in vitro* activities against avian respiratory pathogens (Pirro, 1997; *Norcia et al.*, 1999, Fraatz *et al*., 2002; Himmler *et al.*, 2002; Miller *et al*., 2005). Administered in the drinking water at 10 mg/kg for 5 successive days (the currently labelled dose) enrofloxacin proved efficacious against any bacteria playing a significant role in turkey respiratory disease (Braunius, 1987; Behr *et al*., 1988; Hafez *et al*., 1992; Cargill, 1995; Gautrais and Copeland, 1997; Marien *et al.*, 2006). During this research,

the efficacy of this treatment against respiratory disease in turkeys caused by APV/*O. rhinotracheale* or APV/*E. coli* infections has been endorsed. In every study, *E coli* or *O. rhinotracheale* was rapidly eradicated from the respiratory tract. Already one day after onset of treatment, neither *E. coli* nor *O. rhinotracheale* could be isolated out of the trachea. When turkeys were necropsied immediately after the final treatment day, the respective pathogens were never cultured from the internal organs. The course of respiratory disease was shortened with several days and clinical signs and gross lesions were less severe than those observed in non-treated, *E. coli* or *O. rhinotracheale* infected control groups. It might even be assumed that most of the clinical signs observed originated from the APV inoculation, which was used to trigger the bacterial infections.

Considering PK/PD parameters, the clinical and bacterial outcome of this treatment regimen was expected. Fraatz *et al.* (2006) observed that after continuous drinking medication at a dose of 10 mg/kg body weight in turkeys, steady state serum concentrations of enrofloxacin ranged between 0.32-0.45 µg/ml. In the respiratory tract, concentrations were even 2 times higher. These concentrations exceed MIC_{90} values for *E. coli* (0.06 µg/ml) by approximately four $\log_2$ and for *O. rhinotracheale* (0.25 µg/ml) by two steps (Fraatz *et al.*, 2006). A single oral dose PK study with 10 mg/kg BW enrofloxacin resulted in mean C_{max} values of 1.89 µg/ml and mean $AUC_{0\rightarrow24}$ values of 18.1 h.g/ml (Fraatz, 2006, data on file). Dimitrova *et al.* (2007) observed C_{max}/MIC ratios from 161.23 h to 12.90 h and $AUC_{0\rightarrow24}$/MIC ratios from 2153.44 h to 137.82 h for clinically significant micro-organisms whose MIC varied from 0.008 µg/ ml to 0.125 µg/ml (10 mg/kg BW). Although in the latter study, enrofloxacin was given in a pulsed manner, continuous drinking water treatment is believed to reach similar AUC levels (Stegeman, 1995). Based on the above, a treatment regimen of 10 mg/kg enrofloxacin for 5 continuous days against strains of *O. rhinotracheale* and *E. coli* with MIC of 0.03 µg/ml, should be sufficient to reach C_{max}/MIC ratios >10 and $AUC_{0\rightarrow24}$/MIC ratios >100-125. These ratios are associated with maximal clinical and microbiological effects in Gram-negative bacteria (Blaser *et al.*, 1987; Madaras-Kelly *et al.*, 1996, Frimodt-Moller, 2002).

The dosage of enrofloxacin: time for change?

The dose titration study, administering 5 mg enrofloxacin/kg BW instead of 10 mg/kg BW, continuously in the drinking water for five successive days proved equally effective as the enrofloxacin treatment of 10 mg/kg BW for five continuous days against susceptible *O. rhinotracheale* infections in turkeys (MIC 0.03 µg/ml). Here also, efficacy could have been

predicted. A pharmacokinetic study performed in chickens by Reinhardt *et al*. (2005) in which enrofloxacin concentrations in serum samples were determined after administration of increasing doses of enrofloxacin against *Mycoplasma gallisepticum*, showed that serum concentrations of enrofloxacin are directly proportional to the rate of absorption, which, in turn, is dependent on the concentration at the absorption site. In theory, based on previous C_{max}/MIC ratios and $AUC_{0\rightarrow24}$/MIC ratios, even half the concentration of enrofloxacin in the plasma and respiratory tissues are considered adequate for clinical and microbiological effectiveness against susceptible *O. rhinotracheale* isolates. In the field usually mixed infections occur and first step mutants may be involved in the infection. Still, the efficacy of the 5 mg enrofloxacin/kg BW treatment has not been established against other bacterial respiratory pathogens or less susceptible *O. rhinotracheale* isolates. Hence, it would be advisable to use the 10 mg/kg treatment in respiratory disease outbreaks in the field. Despite their efficacy, previous treatment regimens are considered as cumbersome and time consuming by poultry breeders. Since enrofloxacin is a photosensitive molecule (Sumano *et al*., 2004) the medicated drinking water has to be prepared each day during five days. And, because of the resistance problem associated with this class of antimicrobials, the use of enrofloxacin and other fluoroquinolones in the poultry industry is currently under scrutiny. Although during this entire research period, less susceptible or resistant *E. coli* or *O. rhinotracheale* isolates were never encountered after enrofloxacin treatments of 10 mg/kg BW administered for 5 successive days, other investigators did (Barrow *et al*., 1998, Marien *et al*., 2006a). Isolates with reduced susceptibility or even resistance to fluoroquinolone have also been reported in *Campylobacter sp.* (McDermott *et al*., 2002, Luo *et al*., 2003, Van Boven *et al*., 2003) and *Salmonella sp.* (Barrow *et al*., 1998; Randall *et al*., 2005; 2006), even after single administration of the labelled regimen.

Previous dosing strategies have been designed according PK/PD parameters based on MICs. MIC values, however, refer to drug susceptible populations and are therefore not relevant to the prevention of growth of resistance mutants (Firsov *et al*., 2003; Drlica and Zhao, 2007). In wild type cells, spontaneous mutations in DNA arise at frequencies varying from one in 10^6 to one in 10^9. For MIC measurements, inocula only contain 10^4 cfu (Andrews, 2001). In such relatively small bacterial populations, the occurrence of mutant bacterial isolates is unlikely. However, in diseased animals bacterial populations are more likely the size of 10^7 and 10^8 cfu. Therefore, at the site of infection, bacterial variants with reduced drug susceptibility to enrofloxacin (first-step mutants) will be most likely present (Pourkbahsh *et al*., 1997; Sanders, 2001; Drlica and Malik, 2003; Wetzstein, 2005). Recently the concept of the mutant selection

window hypothesis (Zhao and Drlica, 2008) has found quite some support. The upper boundary of the window, called the mutant prevention concentration (MPC) defines a drug threshold at which visible re-growth from a bacterial population compromising 10^9 to 10^{10} cfu is completely inhibited. Therefore, the heterogenity in sensitivity of the bacterial population at the infection site, which remains undetected in MIC testing, is revealed in MPC curves (Wetzstein, 2005). When a drug falls below the MPC into the so called mutant selection window (of which the lower limit can be approximated by MIC) selection pressure exerted by fluoroquinolone treatment will enrich mutant subpopulations (first step mutants) (Sanders, 2001; Drlica and Malik, 2003; Blondeau *et al.*, 2004). On the other hand, maintaining drug concentrations above the selection window throughout therapy should severely restrict the acquisition of drug resistance. To grow above the MPC, a bacterial isolate would require ≥ 2 concurrent mutations. Since this happens at frequencies of one in 10^{-14} cells, this would be very unlikely (Blondeau *et al.*, 2004). MIC is said to define the sensitivity of the first target. MPC on the other hand defines the sensitivity of the secondary fluoroquinole targets (Wetzstein, 2005).

Resistance to fluoroquinolones arises almost exclusively by *de novo* mutations and is the consequence of gradual accumulation of mutations in the target genes. A first step mutation usually only leads to reduced susceptibility which is regarded as clinically irrelevant (Hooper, 2001; Andriole, 2003). However, when such less susceptible isolates survive in the environment, the MIC of bacterial populations may increase stepwise. Eventually, only 1 additional mutation will be needed before resistance occurs (Schentag *et al.*, 2003). Therefore the selection of less susceptible isolates should be avoided by maintaining drug concentrations above the MPC throughout therapy. To determine whether MPC can be clinically applied, it must be measured for relevant bacterial populations. To be useful, the MPC should be within the concentration limits that can be safely reached in patients.

The possibility has been raised that MPC might be a fixed multiple of MIC (Sanders, 2001). If so, MPC could be determined from MIC measurements. However MPC and MICs correlate poorly (Drlica *et al.*, 2006). For enrofloxacin, MPC's have only been reported for a few pathogens (Etienne *et al.*, 2004; Wetzstein, 2005). For wildtype *E. coli* populations (MIC 0.03), wildtype *E. coli* WT (MIC 0.06) and *E. coli* ATCC 25922 (MIC 0.03), the MPC for enrofloxacin were respectively 0.3-0.35 µg/ml, 0.4-0.5 µg/ml, and 0.15-0.175 µg/ml (Wetzstein, 2005). Since values for *O. rhinotracheale* were not described, the provisional MPC of the *O. rhinotracheale* strain (MIC 0.03 µg/ml) was determined following Blondeau (2001). For this, starter cultures were spread on blood agar plates and incubated 48h at 37°C

in 5% CO_2. Bacterial cells were then transferred from the plates to 500 ml BHI broth (Oxoid) followed by overnight incubation at 37°C in 5% CO_2. After incubation, cultures were estimated to have concentrations of > 3 x 10^8 cfu/ml by turbidity measurements. Cultures were concentrated by centrifugation at 5.000 x g for 10 min and resuspended in 3 ml of PBS. Aliquots of 200 µl, containing $\geq 10^{10}$cfu, were applied to Muller Hinton II agar (Oxoid) dilution plates containing 5% defibrinated horse blood. Agar dilution plates were freshly prepared by incorporating enrofloxacin at thirteen concentrations and dilutions of enrofloxacin (1x to 128x MIC). The fully susceptible LMG ORT strain was included as control. Inoculated plates were incubated for 48h at 37°C in 5%CO_2 and then screened for growth. All plates were re-incubated for an additional 24h and re-examined. MPC was recorded as the lowest antibiotic concentration that allowed no growth. The provisional MPC of *O. rhinotracheale* measured 2 µg/ml. Since the provisional MPC overestimates MPC by about twofold (Blondeau *et al.* 2001), the MPC value of this *O. rhinotracheale* strain is 1 µg/ml. Determinations were made in duplicate, and the results were identical. After continuous drinking medication at a dose of 10 mg/kg body weight in turkeys, steady state serum concentrations of enrofloxacin only ranged between 0.32-0.45 µg/ml (Fraatz *et al.*, 2006). According to the concept of the mutant selection window hypothesis the labelled treatment should not prevent the selection of first step mutations in the *O. rhinotracheale* strains used in this research since enrofloxacin concentrations are too low to exceed *O. rhinotracheales* MPC. Therefore, the treatment regimen in which enrofloxacin was administered at 20 mg/kg BW for 5 successive days, probably better reduces the selection of less susceptible or resistant microbial subpopulations of *O. rhinotracheale*. The increased (doubled) steady-state concentrations in serum and tissues should offer improved conditions maintaining drug concentrations above the selection window throughout therapy. Application of this dosing strategy is, however, not completely straightforward. A five day treatment at 20 mg/kg BW/daily proved only to be equally efficacious as the current labelled dose against *O. rhinotracheale* infections. Improved clinical effect or better bacterial eradication was not noticed. This suggests that drug concentrations in the body were probably higher than necessary for clinical cure against susceptible *O. rhinotracheale* infections. On the other hand, increasing the daily drug dose also means an increase of the treatment cost. In addition, treatment would be still as labour-intensive as the “traditional” labelled dosage regimen.

Rethinking the treatment duration for improved treatment efficacy

To deal with these inconveniences, it was hypothesized that reduced duration therapies could offer a solution. For drugs like enrofloxacin with concentration dependent killing activity higher peak drug concentration in serum and tissues ensure better bacterial eradication and clinical effect, reducing the time needed to treat (Frimodt-Moller, 2002). Therefore administering higher doses of fluoroquinolones might shorten the course of treatment while efficacy is maintained and in the meanwhile the development of resistance in bacteria is reduced (Drusano *et al*., 1993; Forrest *et al*., 1993; Meinen *et al*., 1995, Scaglione *et al.*, 2003, Randall *et al.*, 2006). However, observations made during this research presume that, for an optimal antimicrobial effect, dosing strategies do not only seem to confer appropriate drug concentrations but also an appropriate time of exposure for the target pathogen. Although it has been frequently stated that the antibacterial effects of enrofloxacin at the clinical level are more dependent upon a higher peak in serum concentrations than an extended residence time (Puyt, 1995; Brown, 1996, Riviere, 1999; Sumano and Gutierrez, 2000) the exposure time of enrofloxacin achieved after single day dosing for the treatment of APV/*E. coli* and APV/*O. rhinotracheale* infections was found too short.

Providing the same total dose of 50 mg enrofloxacin /kg BW administered in 5, 10 or 20h proved not to be equally efficacious as the labelled regimen of 10 mg/kg BW for 5 successive days. Although respiratory disease was reduced in time and severity, the clinical and antimicrobial effects of the single day regimens did not match those of the labelled treatment regimen. Moreover, *O. rhinotracheale* and *E. coli* were not completely eradicated from the turkey's respiratory tract, since the pathogens rapidly re-emerged after treatment. Since it is implicit for an optimal dosing strategy that the offending pathogen is eradicated (Tanner, 2003; McKellar et al, 2004) single day treatment regimens are not considered a valuable alternative to the labelled dose. Deplorable, since Randall *et al.* (2006) reported that a single dose of 50 mg enrofloxacin/kg BW in chickens experimentally infected with *Salmonella enterica* might evidence for a decreased selection of less susceptible isolates.

The higher C_{max} achieved with single day dosing, hypothesized to be advantageous compared to the continuous dosing, did not result in improved efficacy against *E. coli* and *O. rhinotracheale* infections in turkeys. Previously, studies comparing pulse enrofloxacin dosing (i.e. administration of the daily dosis enrofloxacin in two to four hour period) and continuous enrofloxacin dosing (administering enrofloxacin in the drinking water over a 24-h period) came to similar observations. In these studies also, higher peak concentrations associated with

pulse dosing were not linked to improved clinical efficacy (Stegeman, 1995; Charleston, 1998; Froyman and Cooper, 2003).

Drug exposure time proved appropriate when the total dose of 50 mg/kg dose was split over two or three days i.e. 2 times 25 mg/kg or 3 times 15 mg/kg, alternated each time by a day without treatment. Both reduced duration medications proved equally efficacious as the labelled regimen in reducing clinical signs, shortening the course of respiratory disease ànd eradicating the target pathogen, e.g. *O. rhinotracheale*. Comparable dosing regimens, splitting the total dose of 50 mg fluoroquinolone/kg BW over 2 or 3 days, tested in humans and chickens against *Salmonella* infections proved also equally efficacious as a 5 day treatment at 10 mg/kg (Hien *et al.,* 1995; Randall *et al.*, 2006). Although only equal and not improved clinical and antimicrobial efficacies were observed - compared to the current labelled treatment regimen - it is believed that these reduced duration medication regimens might have additional advantages. Since the course of treatment is shortened, medicated drinking water will only have to be prepared two or three times instead of five, which is less-time consuming for the turkey breeder. In addition, a regimen of 2 x 25 mg enrofloxacin/kg BW alternated with a non treating day could result overall in a shorter withholding time. Furthermore, the higher peak plasma and respiratory tissue concentrations, resulting from the higher dosages might offer better conditions for restricting selection of less susceptible isolates than the current treatment schedule. This is evidenced by Randall *et al.* (2006), who observed less isolates of *Salmonella enterica* with reduced susceptibility after a 2-day treatment at a dose of 25 mg/kg than after a five day treatment of 10 mg/kg. Therefore, reducing the treatment period against *O. rhinotracheale* infections in turkeys might reduce the rate at which less susceptible *O. rhinotracheale* isolates appear. It is also important to emphasize that the total enrofloxacin dose, compared to current labelled doses, administered was unaltered. Hence, the medication cost is not increased. Therefore, reduced duration medications providing 2 x 25 mg enrofloxacin/kg or 3 x 15 mg enrofloxacin/kg, alternated each time by a day without treatment might be considered as valuable dosing regimens against *O. rhinotracheale* infections in turkeys.

Considerations regarding the safety of the modified regimens, their traits to reduce the emergence of resistance and the used challenge model

Enrofloxacin is regarded as a safe antimicrobial (Anonymous, 2003). In poultry, side-effects have never been observed after administering the registered treatment dose. However, when (daily) antimicrobial drug doses are increased, the risk exists that these higher drug

concentrations might induce toxic effects. Toxic signs sometimes associated with very high enrofloxacin doses in poultry are gastrointestinal disturbances and in one single case arthropathies caused by cartilage lesions have been observed in the past (Walker, 2000). Nevertheless, such symptoms or other adverse events were not observed in any turkey during the studies performed for this research whenever (daily) increased enrofloxacin doses (higher than the labelled 10 mg/kg BW) were administered. Therefore, every treatment regimen tested may be considered safe.

During treatment every enrofloxacin regimen was checked for selection of less-susceptible or enrofloxacin resistant *O. rhinotracheale* or *E. coli* isolates. Since neither the current labelled dosage nor any modified regimen ever promoted the selection of such less susceptible or resistant isolates, it is difficult to conclude if the presumed improved pharmacokinetics and pharmacodynamics of certain modified enrofloxacin regimens (20 mg/kg for five continuous days and the alternative day- reduced duration medications) really reduce emergence of less susceptible or resistant strains than the currently labelled dosis. Applying these regimens in field studies enclosing larger number of birds could result in more conclusive evidence.

When the efficacies of different treatment regimens against a specific disease are to be compared, it is of extreme importance to use a highly reproducible challenge model. If not, it would not be clear whether the differences in clinical and antimicrobial outcome between different treatment groups are due to the impact of the treatment in se or whether they are the result of external factors. This holds true, especially for reproducing a disease as complex as respiratory disease in poultry of which the course and the severity of clinical signs can be influenced by many different environmental factors (Kleven, 2008). Only very recently, Marien (2008) succeeded in the development of such infection model that can reproduce respiratory disease caused by respiratory pathogens currently considered significant in the turkey industry (i.e. APV, *E. coli, O. rhinotracheale*, and *Mycoplasma*). Marien *et al*. (2006) used this infection model to compare the efficacy of different classes of antimicrobials.

Present research shows that the model also proves extremely valuable to compare efficacies of different treatment regimens, since in the currently performed *O. rhinotracheale* trials equivalent results are observed when the clinical scores (Figure 1) and the tracheal titres (Figure 2) of the different positive control groups are compared to each other.

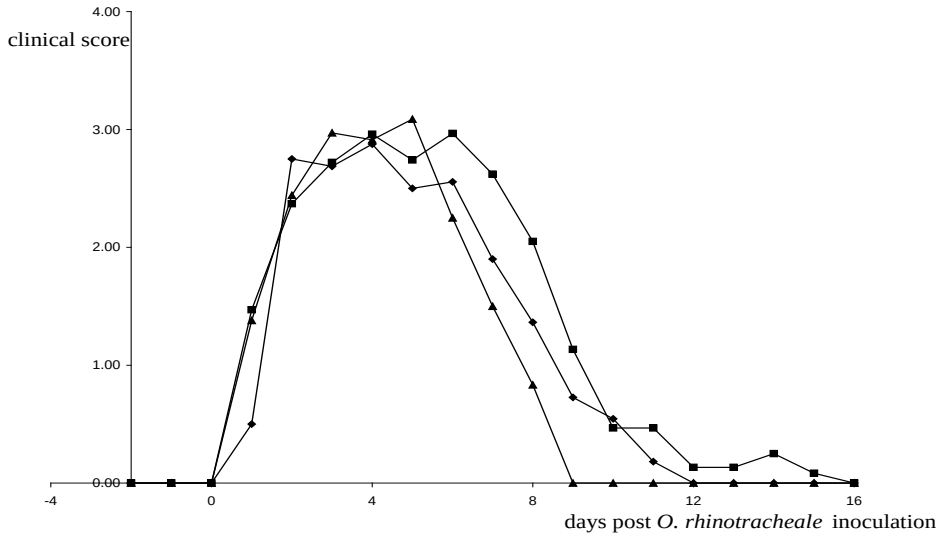

Figure 1. Mean clinical scores in turkeys belonging to the positive control group (i.e. inoculated with APV and three days later with *O. rhinotracheale* without successive enrofloxacin treatment) during the single day treatment study (♦), the dose titration study (■) and the reduced duration medication study (▲).

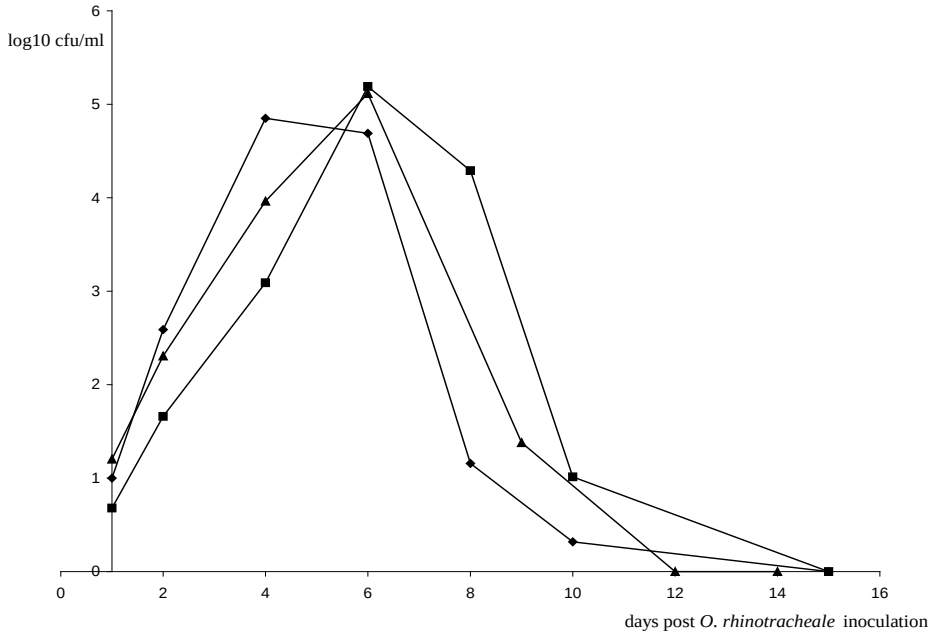

Figure 2. Mean tracheal titres in turkeys belonging to the positive control group (i.e. inoculated with APV and three days later with *O. rhinotracheale* without successive enrofloxacin treatment) during the single day treatment study (♦), the dose titration study (■) and the reduced duration medication study (▲).

Conclusion and recommendations for further research

Overall, the current labelled treatment regimen proves very efficacious against respiratory disease caused by susceptible *O. rhinotracheale* and *E. coli* strains and did not promote the selection of less susceptible or resistant isolates in any study performed during this research. Although not clinically more efficient, certain modified regimens tested, e.g. the alternative day-reduced duration medications could offer a valuable alternative.

However, further work is needed to ascertain how effective these modified treatment regimens are against other bacterial respiratory pathogens not included in this research or mixed bacterial respiratory infections. It would also be interesting to look at the effect of these higher enrofloxacin treatment levels on the gut microbiota, since the selection of resistant mutants during antimicrobial treatment may not only take place at the site of infection but at any bacterial population resident in the body. Furthermore, since evidence exists that less susceptible enrofloxacin isolates are not as clinically irrelevant as primarily thought, it would be useful to test the efficacy of labelled dosage and modified regimens against first step mutants of *O. rhinotracheale* or *E. coli*.

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SUMMARY

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Respiratory disease on turkey farms world-wide leads to economic losses due to growth retardation, increased feed conversion rates, increased mortality rates, higher condemnation rates at slaughter and high medication costs. Bacterial pathogens which play a significant role in the respiratory disease complex are *O. rhinotracheale* and *E. coli*.

To treat respiratory infections in turkeys, drinking water treatment with enrofloxacin is widely used. The current treatment regimen advises a five day treatment at 10 mg/kg body weight. Although this labelled dosage proves very efficacious, the regimen is regarded as cumbersome for many farmers, since the medicated drinking water has to be prepared freshly every day for five days. Besides, *in vivo* emergence of diminished susceptibility or even resistance to fluoroquinolones is commonly encountered in many bacterial species from poultry. Several dosage strategies have been developed for the fluoroquinolone antimicrobials which may increase their efficacy and may reduce the selection pressure for resistance. Nevertheless, controlled clinical studies which address their efficacy against *O. rhinotracheale* and *Escherichia coli* infection have not been reported.

The general introduction starts with a survey on the characteristics of enrofloxacin. The relationship between its structure and activity, the physicochemical properties of the molecule, the mechanism of enrofloxacin action, its pharmacokinetic and pharmacodynamic parameters, related toxic effects and drug interactions, aspects of fluoroquinolone resistance and dosing strategies are the themes that have been reviewed.

Next a review of the literature is given on *O. rhinotracheale* infections in turkeys. Properties described are the general characteristics of *O. rhinotracheale*, the pathogenesis, epidemiology, clinical signs and lesions, diagnosis, preventive measures and treatment. The last part of the general introduction deals with APEC infections in turkeys. The briefy reviewed are the aspects of respiratory colibacillosis in turkeys, the virulence associated factors and diagnosis, treatment and prevention of APEC infections.

The scientific aim of this research was to optimize enrofloxacin dosage regimens against respiratory disease caused by avian metapneumovirus (APV)/*O. rhinotracheale* and APV/*E. coli* infections in turkeys by modifying the duration of the treatments and/or the daily administered enrofloxacin concentrations.

In a first experimental study, three single day enrofloxacin treatment regimens providing 50 mg/kg body weight during a 5, 10 or 20 hour period were compared to the advised multiple day treatment regimen with 10 mg/kg body weight for 5 successive days in turkeys experimentally inoculated with APV and *O. rhinotracheale*. Although initially all dosage regimens cleared *O. rhinotracheale* from the trachea, four days after onset of treatment, *O. rhinotracheale* bacteria were re-excreted in the single day regimens but without clinical relapse. The five day treatment with 10 mg enrofloxacin/kg in turkeys provided the best results for the treatment of an *O. rhinotracheale* infection in growing turkeys by shortening the course and reducing the severity of clinical disease and by eliminating *O. rhinotracheale* from the respiratory tract without re-emergence. None of the treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance.

In a second study, it was assessed whether a single day treatment schedule with 50 mg/kg body weight was clinically equivalent to the advised multiple day treatment schedule with 10 mg/kg body weight for five days against turkeys experimentally inoculated with APV and three days later with *E. coli.* Both the multiple and the single day enrofloxacin treatment regimens reduced *E. coli* multiplication in the respiratory tract tissues (turbinates, trachea and lung) but the five day treatment with 10 mg enrofloxacin/kg in turkeys provided the best results by shortening the course of clinical disease, by eliminating *E. coli* from the respiratory tract without re-emergence, and by reducing macroscopic lesions. None of the treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance.

A third experimental study was a dose titration study. Growing turkeys were first infected with APV and three days later with *O. rhinotracheale*. Twenty four h after *O. rhinotracheale* inoculation, enrofloxacin doses of 5, 10 or 20 mg/kg body weight were administered for 5 successive days. All enrofloxacin treatments were equally efficacious, i.e. equally capable of shortening the course of clinical disease, eliminating *O. rhinotracheale* from the respiratory tract and reducing gross lesions. None of the used treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance to enrofloxacin.

Finally, a reduced duration medication study was performed. To evaluate the current enrofloxacin treatment schedule in growing turkeys experimentally infected with APV and *O. rhinotracheale*, the following enrofloxacin regimens providing the same total administered dose (50 mg / kg BW) were compared: 25 mg / kg BW / day on day 0 and day 2 or15 mg / kg BW / day on days 0, 2 and 4 and 10 mg / kg BW for 5 successive days. Again, all enrofloxacin treatments proved equally efficacious, i.e. equally capable of shortening the

course of clinical disease, eliminating *O. rhinotracheale* from the respiratory tract and reducing gross lesions. None of the used treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance to enrofloxacin.

Based on the results in the experimental studies performed during this research, the current labelled treatment regimen proved very efficacious against respiratory disease caused by susceptible *O. rhinotracheale* and *E. coli* strains. The efficacy of the single day treatments against *O. rhinotracheale* and *E. coli* infections did not equal that of the five day treatment, possibly by not eliminating the respiratory pathogens from the organs, which made it possible for the remaining bacteria to re-emerge. The regimens tested during the dose titration and reduced duration studies proved equally effective against susceptible *O. rhinotracheale* infections. Therefore, depending on the evolution of the respiratory disease in the stable, a flexible adaptation of the duration of enrofloxacin drinking water treatment is possible. The reduced duration applications providing 2 times 25 mg/kg or 3 times 15 mg/kg enrofloxacin, alternated each time by a day without treatment, could offer less time-consuming and equally effective alternatives to the currently labelled treatment against *O. rhinotracheale* infections in turkeys. Neither the current labelled dosage nor any modified regimen ever promoted the selection of less susceptible or resistant isolates.

SAMENVATTING

SAMENVATTING

In de kalkoenindustrie zijn luchtweginfecties wereldwijd een vaak voorkomend probleem met zware economische verliezen als gevolg. De verliezen zijn voornamelijk te wijten aan verhoogde voederconversie, vermindere groei, verhoogde sterftepercentages, een hoger aantal karkasafkeuringen bij verwerking en hogere medicatie kosten. Twee bacteriële ziektekiemen die vaak geassocieerd worden met ademhalingsproblemen bij kalkoenen zijn *Ornithobacterium rhinotracheale* en *Escherichia coli*.

Enrofloxacine is een antimicrobieel product, behorend tot de klasse van de fluoroquinolones, dat vaak wordt voorgeschreven ter behandeling van zulke ademhalingsinfecties in kalkoenen. Het huidig behandelingsschema adviseert een dosis van 10 mg enrofloxacine/kg lichaamsgewicht, toe te dienen via het drinkwater gedurende 5 opeenvolgende dagen (label dosering). Ondanks zijn bewezen doeltreffendheid, vinden veel kalkoenhouders dit behandelingsschema omslachtig omdat het gemedicineerd drinkwater elke dag -vijf dagen lang- opnieuw moet worden aangemaakt. Ook worden er *in vivo* uit pluimvee regelmatig bacteriën geïsoleerd die een verminderde gevoeligheid of zelfs resistentie tegenover fluoroquinolones vertonen. Recent zijn er echter verscheidene dosisstrategieën ontwikkeld die de werkzaamheid van deze klasse antimicrobiële middelen zouden kunnen verhogen en daarnaast de selectie van resistente kiemen zouden verminderen. De efficaciteit van deze strategieën zijn echter nog nooit uitgetest op *O. rhinotracheale* en *E. coli* infecties.

In de algemene introductie van dit werk wordt eerst enrofloxacine onder de loep genomen. De structuur, de fysicochemische eigenschappen, het werkingsmechanisme van de molecule, de farmacodynamische en farmacokinetische eigenschappen, de toxische effecten, de interacties met andere preparaten, resistentievorming en de ontwikkelde dosisstrategieën van het antimicrobieel product worden besproken.

Vervolgens wordt er een literatuuroverzicht gegeven over *O. rhinotracheale* infecties in kalkoenen. De algemene kenmerken, de pathogenese, de epidemiologie, klinische symptomen en klinische letsels, diagnose, de behandeling van *O. rhinotracheale* infecties en preventieve maatregelen komen aan bod. Tenslotte volgt er een stukje over Aviaire Pathogenische *E. coli* (APEC) infecties in kalkoenen, met daarin de volgende onderwerpen: respiratoire colibacillose, de geassocieerde virulentie factoren en de diagnose, de behandeling en de preventie van APEC infecties.

Het doel van dit onderzoek was om het huidige behandelingsschema van enrofloxacin dat gebruikt wordt ter behandeling van APV/*O. rhinotracheale* and APV/*E.coli* infecties te optimaliseren door de behandelingsduur en/of de dagelijks toegediende dosis van enrofloxacine aan te passen.

In een eerste studie werd de efficaciteit van eendaagse enrofloxacine behandelingen vergeleken met die van het huidig geregistreerde meerdaagse behandelingsschema. Na inoculatie met APV en *O. rhinotracheale* werden drie groepen kalkoenen gedurende 5, 10 of 20 u behandeld met een dosis van 50 mg enrofloxacine/kg lichaamsgewicht. Een vierde groep kreeg gedurende 5 opeenvolgende dagen 10 mg/kg enrofloxacine toegediend. Alle behandelingen slaagden erin *O. rhinotracheale* uit de trachea te verwijderen, maar enkele dagen na start van de medicatie werd na de eendags-therapieën O. *rhinotracheale* opnieuw in de trachea aangetroffen. Van de vier behandelingen, slaagde de huidig geregistreerde behandeling er het beste in het ziekteverloop in te korten en het ziektebeeld te verzachten zonder re-excretie van *O. rhinotracheale*. Tijdens de studie werden geen *O. rhinotracheale* isolaten geïsoleerd met verminderde gevoeligheid of resistentieontwikkeling tegenover enrofloxacine.

In een tweede studie werd onderzocht of één dag behandelen aan een dosis van 50 mg enrofloxacine/kg lichaamsgewicht even werkzaam is als de huidig geregistreerde behandeling van 10 mg enrofloxacine/kg gedurende 5 opeenvolgende dagen voor de behandeling van APV/*E. coli* infecties in kalkoenen. Beide behandelingen reduceerden de *E. coli* vermenigvuldiging in de luchtwegen, maar de geregistreerde behandeling slaagde er het best in *E. coli* uit de kalkoenen te elimineren, de ziekteduur te verkorten, en de symptomen en letsels te beperken. Tijdens de studie werden geen *O. rhinotracheale* isolaten geïsoleerd met verminderde gevoeligheid of resistentieontwikkeling tegenover enrofloxacine.

Vervolgens werd een dosis-titratie studie uitgevoerd. Na inoculatie met APV en *O. rhinotracheale* werden kalkoenen 5 dagen na elkaar behandeld met 5, 10 of 20 mg enrofloxacine/kg lichaamsgewicht/dag. Alle behandelingen bleken even effectief in het elimineren van O. *rhinotracheale* uit de luchtwegen, het verkorten van de ziekteduur, en het verminderen van klinische symptomen en letsels. Tijdens de studie werden geen *O. rhinotracheale* isolaten geïsoleerd met verminderde gevoeligheid of resistentieontwikkeling tegenover enrofloxacine.

Tenslotte werd een studie uitgevoerd waarin het huidige geregistreerde behandelingsschema van enrofloxacine (10 mg/kg gedurende 5 opeenvolgende dagen) werd

vergeleken met therapieën waarbij op alternerende dagen werd behandeld met behoud van de totale dosis: 25 mg/kg/dag op dag 0 en dag 2 of 15 mg/kg/dag op dag 0, 2 en 4. Ook in deze studie bleken alle behandelingen even effectief in het elimineren van O. *rhinotracheale* uit de luchtwegen, het verkorten van de ziekteduur, en het verminderen van klinische symptomen en letsels. Tijdens de studie werden geen *O. rhinotracheale* isolaten geïsoleerd met verminderde gevoeligheid of resistentieontwikkeling tegenover enrofloxacine.

Uit de resultaten van de uitgevoerde experimenten kunnen we besluiten dat het huidige geregistreerde behandelingschema van enrofloxacine (10 mg/kg gedurende 5 opeenvolgende dagen) zeer effectief is tegen ademhalingsinfecties veroorzaakt door APV/*O. rhinotracheale* en APV/*E. coli*. Behandelingsschema's waarin de totale enrofloxacine dosis (50 mg) in éénzelfde dag wordt toegediend bleken geen goed alternatief, aangezien zowel *O. rhinotracheale* als *E. coli* niet volledig werden geëlimieerd en na enkele dagen opnieuw konden vermenigvuldigen in de luchtwegen van de kalkoenen. De behandelingsschema's die werden getest tijdens de dosis titratiestudies en de medicaties waarbij op alternerende dagen werd behandeld bleken even effectief als het huidig geregistreerde regime. Dit wijst erop dat, afhankelijk van het verloop van de ziekte in de stal, drinkwater therapie met enrofloxacine flexibel kan worden aangepast. Bovendien leveren de therapieën waarbij 2 keer 25 mg/kg of 3 keer 15 mg/kg enrofloxacine wordt toegediend een waardevol alternatief voor het geregistreerde regime, aangezien maar 2 of 3 dagen moet behandeld worden in plaats van 5.

CURRICULUM VITAE

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An Garmyn werd op 29 juni 1980 geboren te Antwerpen. Na het beëindigen van haar studies secundair onderwijs, richting Latijn-Wetenschappen, aan het sint-Michiels college te Brasschaat, startte ze in 1998 met de studies Diergeneeskunde aan de Universiteit van Gent. In 2006 studeerde ze met onderscheiding af als dierenarts.

Vanaf september 2006 trad zij in dienst als wetenschappelijk medewerker bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten. Gedurende drie jaar werkte zij aan een onderzoeksproject “Enrofloxacine medicaties ter behandeling van respiratoire infecties bij kalkoenen”, dat gesteund werd door Bayer HealthCare, Animal Health.

An Garmyn is auteur van meerdere publicaties in internationale tijdschriften.

PUBLICATIONS

PUBLICATIONS

Garmyn, A., Martel, A., Froyman, R., Nauwynck, H., Duchateau, L., Haesebrouck, F. and Pasmans, F. (2009) Efficacy of four enrofloxacin treatment regimens against experimental infection in turkey poults with avian pneumovirus and *Ornithobacterium rhinotracheale*. *Avian Pathology*, 38, 287-292.

Garmyn, A., Martel, A., Froyman, R., Nauwynck, H., Duchateau, L., Haesebrouck, F. and Pasmans, F. (2009) Effect of multiple and single day enrofloxacin medications against dual experimental infection with avian pneumovirus and *Escherichia coli* in turkeys. *Poultry science, 88,* 2093-2100.

Garmyn, A., Martel, A., Froyman, R., Ludwig, C., Nauwynck, H., Haesebrouck, F. and Pasmans, F. (2009). The effect of reduced treatment time and dosage of enrofloxacin on the course of respiratory disease caused by avian pneumovirus and *Ornithobacterium rhinotracheale*. *Poultry Science, 88,* 2315-2323.

DANKWOORD

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