A comparative study of the minimum inhibitory and mutant prevention concentrations of florfenicol and oxytetracycline for animal isolates of *Pasteurella multocida* and *Salmonella* Typhimurium

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PREFACE

Declaration by Student

I, Jeanette Maria Wentzel declare that this dissertation is my own work, carried out originally under the supervision of Prof M. van Vuuren of the University of Pretoria and is in accordance with the requirements of the University for the degree Magister Scientiae (Veterinary Tropical Diseases). Prof. M. van Vuuren served as supervisor during the project.

Date

Signature

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ABSTRACT

This study was undertaken to compare the MIC (minimum inhibitory concentration) and MPC (mutant prevention concentration) values for oxytetracycline and florfenicol against strains of *Pasteurella multocida* isolated from cattle and pigs, and for enrofloxacin against strains of *Salmonella* Typhimurium isolated from horses.

Isolates of *P. multocida* from cattle and pigs, and *S.* Typhimurium from horses were obtained from specimens or isolates from contributing laboratories. All the equine isolates and 50% of the cattle and pig isolates were from clinically sick animals. All isolates were tested in duplicate with both the MIC and the MPC methods. The MIC method used was the standardized microdilution method performed in microtitre plates. The MPC method used was according to the method described by Blondeau. This method was modified, to make use of smaller plates and lower volumes of antimicrobials, but retaining a final bacterial concentration of 10^9 colony-forming units per ml.

The antimicrobials were dissolved as described in the certificates of analyses. Enrofloxacin and oxytetracycline were dissolved in water, and florfenicol was dissolved in alcohol. For the MPC method, an additional control was added to one quadrant of a four-quadrant 90mm plate/petri dish. The antimicrobials were tested as individual antimicrobials and not as combinations. Both the MIC and MPC methods included ATCC (American Type Culture Collection) strains as control organisms and were evaluated according to the guidelines of the CLSI (Clinical and Laboratory Standards Institute).

The MIC_{50} values for enrofloxacin against *Salmonella* Typhimurium isolates from horses was 0.25 µg/ml and the MPC_{50} values 0.5 µg/ml. A comparative reference range was not available as enrofloxacin is not registered in South Africa for use in horses, and is used extra-labelly. The results for florfenicol against *P. multocida* yielded an MIC_{50} value of 0.5 µg/ml and an MPC_{50} value of <2 µg/ml. The close relationship of these two concentrations is an indication of the effectiveness of florfenicol when used against *P. multocida.* The PD/PK data with a value of 141.78 for AUC/MIC provided additional support for the efficacy of florfenicol against *P. multocida*. The PD/PK value of >125, is an effective parameter for treatment of Gram-negative bacteria. The corresponding results for oxytetracycline were above the MIC value but fell within the mutant selection window. The results point to the fact that the use of oxytetracycline against *P. multocida* may not be effective in preventing the appearance of first step mutant strains when used at current recommended dosages. The PK/PD data, using AUC/MIC, yielded a value of 56. Some of the isolates (55.17%) had an MPC value of 16 µg/ml.

Whereas the MIC method is used routinely in diagnostic laboratories, the MPC method can be employed to generate data that can be applied where antimicrobial treatment of certain bacteria is problematic and standard

treatment may lead to the development of resistance. Data obtained from such studies will enable manufacturers of antimicrobial drugs to adapt antimicrobial therapy where practical and feasible to prevent the development of first step mutants.

LIST OF ABBREVIATIONS

ATCC	American type culture collection
CLSI	Clinical Laboratory Standards Institute
MIC	Minimum inhibitory concentration
MIC_{50}	The concentration of an antimicrobial agent which will inhibit 50% (half) of the isolates tested against the antimicrobial drug
MIC_{90}	The concentration of an antimicrobial agent which will inhibit 90% of the isolates tested against the antimicrobial drug
MH media/agar	Mueller Hinton media/agar
FFC	Florfenical, a fluorinated chloramphenicol derivative, only used in veterinary medicine
MPC	Mutant prevention concentration
PK	Pharmacokinetics
PD	Pharmacodynamics
AUC	Area under the curve
BRD	Bovine respiratory disease

CHAPTER 1

1. INTRODUCTION

1.1 Motivation for the Research Project

Resistance of bacteria to antimicrobial drugs is a global problem that also influences the veterinary profession. It influences the dosing regimens and effective dosing volumes of antimicrobial drugs administered to animals. Current laboratory methods for determination of the susceptibility of bacteria to antimicrobial drugs have shortcomings with respect to detection of bacteria that may have reduced susceptibilities. Such bacteria may survive treatment and develop into resistant strains.

The mutant prevention concentration (MPC) is a relatively new method to test the susceptibility of organisms to antimicrobial drugs and has been proposed as an alternative to the MIC as a measure of antimicrobial activity. The MPC method is performed on plates with different concentrations of antimicrobial drugs added, thus being able to test various antimicrobial concentrations in the same time frame. In addition, the MPC is determined at a bacterial concentration of 10^9 colony forming units (CFU)/ml.

Maintaining the antimicrobial concentrations above the MPC will theoretically prevent the selection of resistant organisms. Concentrations that are maintained in the range between the MIC and MPC [the mutant selection window (MSW)] are thought to promote the selection of resistant subpopulations. When MPCs exceed MICs, it does not imply that therapeutic doses should automatically be increased. Several outcomes will have to be evaluated and include *inter alia* the increased withdrawal period for meat products, the implications for safety of food products for consumers, the ability to achieve the MPC values in target tissues, and the possibility of tissue toxicity in the recipient animals.

Theoretically, the MPC when validated for treatment will enable the practitioner to reduce the chances of unknowingly selecting for antimicrobial resistance, since the MPC prevents first steps mutants, while the MIC is the concentration that inhibits the wild strain of an organism.

Combined MIC and MPC values have so far been determined for only a few bacterial pathogens isolated from animals, and similar studies have not been conducted in South Africa. The information obtained from this study will make veterinary practitioners and the pharmaceutical industry aware of new approaches to address the development of resistance to antimicrobials and encourage the prudent use of these valuable drugs.

Currently most laboratories make use only of the disk diffusion (Kirby Bauer method) that provides results to practitioners as sensitive, intermediate or resistant. The Kirby Bauer method utilizes impregnated disks that limit each antimicrobial drug included in the test to a single concentration per disk.

An alternative method is the agar dilution method that provides a specific minimum inhibitory concentration (MIC) of an antimicrobial drug. The antimicrobial drug is added at a known concentration into the agar contained in a plate. A standard concentration of the pathogen is inoculated onto the surface of this medium. The agar plates are incubated and examined for bacterial growth. No growth of the test organism indicates that it is susceptible to the known antimicrobial concentration incorporated into the medium.

The MIC broth dilution method is performed in 96-well microtitre plates and is a quantitative method that makes use of breakpoint values to place an organism in either a sensitive or a resistant category. Each plate is set up according to the CLSI (Clinical Laboratory Standards Institute) guidelines, and plates can be designed for use for a specific species or for testing specific bacterial organisms.

The aims of this study were:

- To determine the MIC and MPC values of selected antimicrobial drugs against strains of *Salmonella* Typhimurium isolated from horses and of *Pasteurella multocida* strains isolated from cattle and pigs in South Africa;
- To generate data on MIC and MPC values that could be used by researchers and pharmaceutical companies to determine optimal doses for treatment of food-producing animals.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Background

The need for the effective antimicrobial treatment of bacterial diseases in animals, and use of antimicrobial drugs in agriculture, food production and veterinary science has been identified (Blondeau, 2009a, Caprioli, Busani, Martel & Helmuth, 2000). The efficacy of treatment is hampered by bacterial resistance and effective testing procedures, as well as the lack of control measures for the use of antimicrobials in agriculture (Zhao & Drlica, 2001). The resistance of bacterial organisms to available antimicrobial drugs is of increasing concern in both veterinary and human medicine (Blondeau, 2009a). The resistance of food-borne pathogens such as *Salmonella* spp. holds a great risk for the future, since the same active ingredients are used in the treatment of animal and human infections. Resistant bacteria may be transferred to humans by contact or food contamination (Schwarz, Kehrenberg & Walsh, 2001; Byarugaba, 2004). This leads to an economic and medical problem, as more than half of all antimicrobials used globally are used in the food animal industry (Aarestrup, 1999; Teuber, 2001).

The WHO (World Health Organization) and OIE (World Organisation for Animal Health) compiled a list of antimicrobials that are seen as critically important, highly important and important. Aminoglycosides, cephalosporins, macrolides, penicillins, phenicols (florfenicol), quinolones (enrofloxacin), sulfonamides and tetracyclines (oxytetracycline) are all critically important. Rifamycins, fosfomycin, lincosamide, pleuromutilins and polypeptides are classed as highly important. Bicyclomycins, fusidic acids, novobiocins, orthosomycins, quinoxalines and streptogramins are classed as important (Food and Agriculture Organization, WHO and OIE; 2008). The compilation of such a list underpins the importance that international organizations attach to antimicrobial drugs and the threat of resistance to these drugs.

The value of the MPC method lies in the fact that it will help to increase the therapeutic efficacy of antimicrobials used in clinically sick animals. It will contribute to a reduction in the development of resistance of micro-organisms and prevent development of first-step mutants of the organism. MPC methods will therefore improve treatment regimens (Blondeau, Xilin, Hansen & Drlica, 2001; Burch, 2007; Blondeau, 2009a; Zhao & Drilca, 2008). In a study conducted during 2003, the antimicrobial drug based on MPC values killed the wild strains of organisms and prevented development of any further resistant mutant organisms, e.g. enrofloxacin against *Escherichia coli* infections in pigs (Drlica, 2003).

Blondeau (2009b) foresees that the MPC values will lead to the use of higher concentrations of antimicrobials, but over a shorter period. In practice, this will lead to the use of single injection, short acting antimicrobial drugs. On the other hand, when using the MIC values, lower concentrations of antimicrobials are used for longer

periods. An example of the application of this concept for the treatment of animals is the use of high dose marbofloxacin for the treatment of bovine respiratory disease (BRD).

The MPC method is described in Figure 1. This method is more labour intensive and needs additional preparation before the test can be run. At least three agar plates are used per organism. After overnight incubation, the growth is transferred to new media to enhance the growth of the organisms. This is followed by a centrifugal step to concentrate the organisms. The samples are then resuspended and added to agar plates with different concentrations of antimicrobials. An important feature of the method is the final testing concentration of the isolate of 10^9 CFU/ml as seen in step 4 of Figure 1.

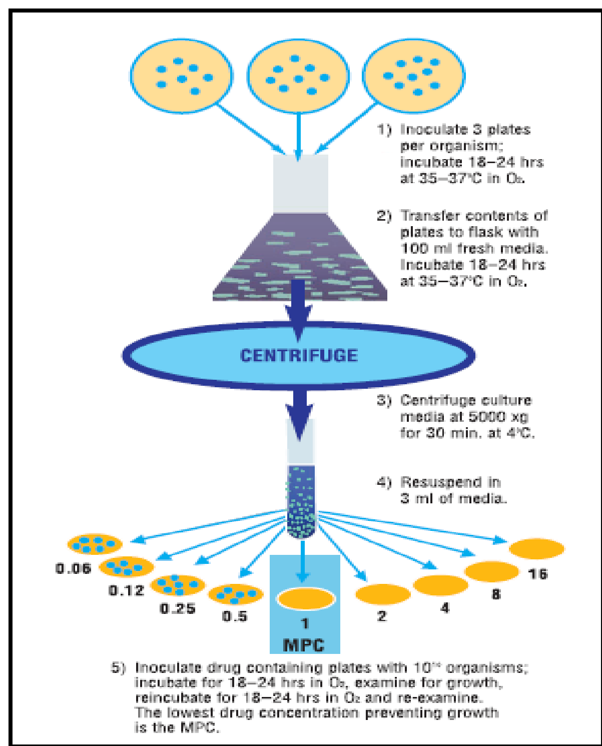

Figure 1**: MPC method according to Blondeau, 2009a**

The MPC method has been used mostly for fluoroquinolones, although later beta-lactams were also included in testsing (Smith, Nichol, Hoban, & Zhanel, 2003). However, some researchers feel that the use of MPC method should be limited to fluoroquinolones only. In one study all the different antimicrobial classes were tested and inaccuracies or discrepancies were found when the MPC testing was used to determine primary mechanisms of resistance (Smith *et al*., 2003). Other disadvantages are that MPC method results will be less valuable for patients with normal intact immune systems, since for animals with normal functioning immunity, both susceptible and resistance bacteria are likely eliminated. It will also not yield optimal results when used in immuno-compromised patients that have had prior infections or prior exposure to an antimicrobial, or in which therapy for acute infections failed, since resistant subpopulations may continue to proliferate and heighten the possibility of second step mutants occurring (Blondeau, 2012).

The MPC value is estimated as the drug concentration that blocks bacterial growth at a concentration of 10^9-10^{10} colony forming units (CFU) per ml, when applied to agar or tested in liquid medium. Concentrated inocula ensure the presence of mutant subpopulations; consequently, the MPC estimates resistant subpopulation susceptibility. The MPC can also be defined as the MIC required to block the growth of the most resistant first-step mutation(s) in a heterogeneous bacterial population (Metzler, Hansen, Hedlin, Harding, Drlica & Blondeau, 2004; Smith *et al.*, 2003).

The difference between the MIC value and the MPC value of an isolate is explained in Figure 2. The figure depicts the basic differences between the two methods by means of the mutant selection window. The MIC is reflected as a concentration of 4 µg/ml and the MPC a concentration of 16 µg/ml. The area between the MIC and MPC values is known as the mutant selection window. This is the area where mutant fractions of bacterial populations are enriched.

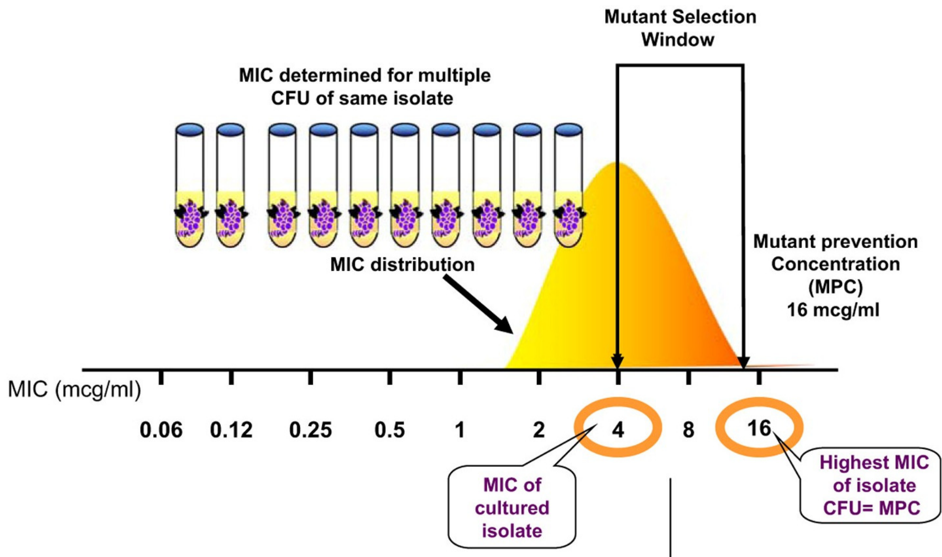

Figure 2: **Illustration of the difference between MIC and MPC concentrations and the role of the mutant selection window (Booth, 2006).**

The first MIC method was introduced in the 1960's by the company Eli Lily. The MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a micro-organism after 24 hours incubation in comparision to the control wells. The MIC determination makes use of a concentration of 10^4–10^5 bacteria per well in the microtitre plates (Metzler *et al.,* 2004; Smith *et al.*, 2003). The advantages of MIC testing are that the method is relatively straightforward, easy to prepare and the test results are repeatable. The method uses only a limited volume of antimicrobials and is fairly cheap. If prepared plates are used little or no preparation is needed and tests can be completed within a short turnaround time. The disadvantages are that the test results can differ

with a small variation in the inoculum size (lower inoculation will make an MIC result lower) and also with variation in incubation time (longer incubation will make the MIC higher).

The practitioner can only use an antimicrobial against an organism if they know the mechanism of action for the chosen antimicrobial and if it works as a bactericidal (the antimicrobial's ability to kill) or a bacteriostatic (the inhibition of microbial growth) drug (Booth, 2006). The bacterial action and mechanism of action play an important role as pharmacodynamic parameters of an antimicrobial. In terms of pharmacokinetic parameters, the activity can be either time dependent; (the antimicrobial has antibacterial activity in the time that the drug concentration is above the MIC value) or concentration dependent (linked to the drug concentration above the MIC value).

In terms of the antimicrobial drugs used in this project, enrofloxacin is a fluoroquinolone antimicrobial. It has good tissue penetration attributes and can be used against Gram-positive and Gram-negative organisms. Even though it is not registered for use in horses, practitioners do use it (Langston, Sedrish & Booth, 1996). The mechanisms of resistance of bacteria against enrofloxacin are target site mutation, decreased permeability, efflux and target site protection with a bacteriostatic as well as bactericidal activity (CLSI, 2008). The bactericidal effect of enrofloxacin is concentration-dependent and the pharmacodynamic (PD) parameter used to evaluate the activity is AUC (area under the curve)/MIC. Enrofloxacin has both concentration- and time-dependant activities (Martinez & Silley, 2010).

Florfenicol is a fluorinated chloramphenicol derivative used in veterinary medicine. It is predominantly used in large animals. The main organisms targeted by this antimicrobial are the BRD group of organisms (Priebe & Schwarz, 2003). Florfenicol is a broad-spectrum synthetic antibiotic from the family of phenicols active against most Gram-positive and Gram-negative bacteria isolated from domestic animals. Florfenicol acts by inhibiting protein synthesis in the ribosome and is bacteriostatic. However, bactericidal activity has been demonstrated *in vitro* against *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* when it is present at concentrations above the MIC for 4 to 12 hours. The phenicol group of antimicrobials to which florfenicol belongs binds to the peptidyl transferase region of the ribosomal RNA of the 50S ribosomal subunit. This interaction is limited to ribosomal RNA and does not involve ribosomal proteins. Bacterial resistance to florfenicol includes mechanisms of action such as decreased permeability, and antimicrobial efflux pumps. The antibacterial action of florfenicol is time-dependent and is characterized by T>MIC (the time the drug concentration remains in excess of the MIC) (Martinez *et al.,* 2010).

Oxytetracycline is used for the treatment of respiratory infections in animals. This broad-spectrum antimicrobial drug is also used for the treatment of *Chlamydophyla* infections, eye infections and genital infections. Mechanisms of resistance against oxytetracyclines include efflux pumps, ribosomal protection during detoxification and target site mutation. The activity of oxytetracycline is bacteriostatic and time-dependent (Martinez *et al.,* 2010).

A number of guidelines can be implemented to improve the optimum use of antimicrobial drugs available in veterinary medicine. These include proper surveillance or monitoring systems as well as new methods for the detection of antimicrobial susceptibility of organisms (Byarugaba, 2004). The WHO recommends that antimicrobials used in animals should be regulated and that surveillance for the presence of resistance and the use of antimicrobials must be maintained. They also recommend the banning or phasing out of growth promoters and increasing and promoting the education of farmers and veterinary practitioners with regard to antimicrobial use (Okeke, Klugman, Bhutto, Duse, Jenkins, O'Brien, Pablos-Mendez & Lazminarayan, 2005). Lovemore (2005) stated that besides the prudent use of antimicrobials and the pressures associated with the emergence of more resistant organisms, pharmaceutical companies need to re-invest in the production of new antimicrobials.

European Union countries started programmes to monitor antimicrobial resistance (Gnanou & Sanders, 2000). Different countries decided on different methods, resulting in several reference systems. These include: National Committee of Clinical and Laboratory Standards (NCCLS), Comite de l'antibiogramme-Societe de microbiologie (CA-SFM), the British Society for Antimicrobial Chemotherapy (BSAC), the Swedish Reference Group for Antimicrobials (SRGA) and lastly the Deutsche Institute fur Normung (DIN). This created different breakpoints and reference systems, but most of the systems are based on the disk diffusion method, with reference ranges being similar (Gnanou & Sanders, 2000). Breakpoints refer to the critical drug concentrations that characterize specific antibacterial activities (Denis, *et al*., 2009).

2.2 Applicable antimicrobial resistance research

Many studies compared MIC and Kirby Bauer method results. The Kirby Bauer method was used in numerous studies in comparison with the MIC method to prove the efficacy and sensitivity of the MIC method. By comparing the agar disk diffusion and microdilution methods, the results revealed a 90% or higher correlation for streptococci and staphylococci, and a correlation percentage of 95.8% for *Pasteurella* (Rerat, Albini, Jaquier & Hussy, 2012). Priebe and Schwarz (2003) also compared the disk diffusion and microdilution methods for *P. multocida* isolates from both bovine (122) and porcine (212) samples against florfenicol. The results showed that the MIC_{90} was 0.5 µg/ml with a disk range of 30-47 mm in cattle, while the MIC_{90} for porcine samples was 0.5 µg/ml with a disk range of 28-43 mm, indicating that the MIC and zones of inhibition are similar and that no resistance existed during the study.

Some of the published studies on MIC methods applicable to this project include a 4-year-survey of isolates of BRD in North America (Watts, Yancey, Salmon and Case, 1994). They determined the MIC of isolates of *Pasteurella haemolytica*, *Pasteurella multocida* and *Histophilus somni* to various antimicrobial agents. The results showed that *P. haemolytica* (461 isolates) had a 100% susceptibility to ceftiofur and only a 5.4 % susceptibility to erythromycin. *P. multocida* (318 isolates) had a 100% susceptibility to ceftiofur and the lowest

susceptibility of 16% to erythromycin. *H. somni* (109 isolates) had the best overall susceptibility to all the antimicrobial agents, with a 100% susceptibility to ceftiofur and a susceptibility of 35.8% to sulfamethazine. The study achieved the objective to indicate the susceptibility of bovine respiratory pathogens to antimicrobials by means of MIC values.

Rerat et al, (2012) conducted a specific study on the treatment and antimicrobial resistance of members of the *Pasteurellaceae*. The study was done on 60 veal calves with respiratory problems purchased from 22 different farms. The complete treatment histories for all the animals were available and none were vaccinated against BRD. Trans-tracheal lavage samples were collected and tested. The researchers also enriched the Mueller Hinton broth used in the microtitre plates with lysed horse blood. The *Pasteurellaceae* showed no resistance against both florfenicol (MIC ≤2 µg/mL) and gentamycin.

In a European study, 6 countries participated over a 3 year period and each country tested between 109 and 504 isolates of *P. multocida*. A decrease in resistance was found against ampicillin, tetracyclines and sulphonamides in the Netherlands, England, Wales, France and Denmark (Hendriksen, Mevius, Schroeter, Teale, Meunier, Butaye, Franco, Utinane, Amando, Moreno, Greko, Stark, Berghold, Myllyniemi, Wasyl, Sunde & Aarestrup, 2008).

Giguere and Tessman (2011) pointed out that MIC measures only the inhibition of bacterial growth for the specific organism and not the killing of the pathogen as an endpoint value. They also mentioned that there is a lack of species-specific data between MIC and *in vivo* infections. Some of the veterinary organisms do not have any references or CLSI guidelines, therefore the human guidelines are used for the interpretation of veterinary organisms (Hesje, Tillotson & Blondeau, 2007). As an alternative small animal references are used for large animal veterinary organisms (Giguere *et al*., 2011).

During a study in 2007, MPC methods were compared to molecular-based methods such as PCR (polymerase chain reaction) methods or used in conjunction with PCR. The bacterial concentration at the MPC value was analysed with quantitative PCR methods, specifically PCR mapping and sequencing. The PCR methods showed that the *S.* Typhimurium isolates had mutations on the gene codons 81, 83 and 87 against fluoroquinolones (Pasquali & Manfreda, 2007).

Blondeau and various other researchers did numerous studies comparing MIC and MPC methods (Blondeau, Borsos, Blondeau, Blondeau & Hesje, 2007a). In 2007 a correlation study was done between MIC and MPC of enrofloxacin, florfenicol, tilimicosin and tulathromycin against *M. haemolytica* collected from cattle with BRD (Blondeau, Borsos, Blondeau, Blondeau & Hesje, 2007a). Not only did the study rank and measure the MIC and MPC values but also calculated the pharmocodynamics(PD)/pharmocokinetics(PK), ranking enrofloxacin as the

most potent and tulathromycin as the least potent, according to their MIC values. This study concluded that treatment administered above MPC values would reduce the amplification of resistant bacteria.

The same researchers did a concentration-dependent kill study with enrofloxacin with the use of MIC, MPC, maximum serum and tissue drug concentrations. The enrofloxacin performed better at higher concentrations, since it is concentration-dependent, thereby reducing the risk of resistance development. The enrofloxacin had bactericidal activity against the inocula at a concentration of 10^5-10^9 colony forming units/milliliter (CFU/ml) (Blondeau, Borsos, Blondeau, Blondeau & Hesje, 2007b).

Besides comparing the MIC and MPC values, some researchers also did correlation studies between the methods, with the objective of proving that the MPC value is either 2-fold, 4-fold or any-fold of the MIC value. However, this was not true for *Streptococcus* and *Pseudomonas* spp. with the aid of a quinolone study (Blondeau, 2009a, Zhao & Drlica, 2008). According to Drlica, Zhao, Blondeau and Hesje (2006), a low correlation between the MIC and MPC will have a negative influence on treatment of individual patients, but it can be expected with clinical studies, due to specific inclusion criteria.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

Strains of *S.* Typhimurium and *P. multocida* isolated from specimens submitted by state and private veterinary practitioners were obtained from Idexx Laboratories, Dept. of Veterinary Tropical Diseases, University of Pretoria, Disease Control Africa, Pathcare Veterinary Laboratories, Vetdiagnostix and Stellenbosch Provincial Veterinary Laboratory. These organisms were stored frozen at -70 ºC.

A total of twenty seven *Salmonella* Typhimurium and twenty nine *Pasteurella multocida* strains were included for testing.

Table 1: **Number of samples, source and species from which the isolates were obtained**

Pasteurella multocida			***Salmonella* Typhimurium**		
No. samples	**Species**	**Source**	**No. samples**	**Species**	**Source**
16	Bovine	Trans-tracheal aspirate	8	Equine	Joint
9	Bovine	Lung	14	Equine	Faeces
4	Porcine	Lung	1	Equine	Blood culture
			3	Equine	Abscess
			1	Equine	Bone

3.2 Identification of *Salmonella* Typhimurium and *Pasteurella multocida*

The isolates were confirmed as either *P. multocida* or *S.* Typhimurium, by means of biochemical methods, (refer to Tables 2 and 3) (Songer, & Post, 2005; Quinn, Carter, Markey & Carter, 1994) or the Vitek system (Biomerieux)(Vitek 2XL, France). Vitek is an automated microbiology system using growth-based technology and colorimetric reagent cards that are incubated and interpreted automatically. Various methods as listed in Table 2 were used to confirm the identity of the *S.* Typhimurium isolates, including Gram's stain and polyvalent antisera for flagellar (H) and Somatic (O) antigens. (polyvalente antisera, Biorad)

Table 2*:* **Tests used to identify *Salmonella* Typhimurium**

Tests	Result
Growth on selective media	Black colonies on XLD and red colonies on selenite broth
Growth on McConkey agar	No lactose fermentation
Haemolysis on blood agar	Negative
Lysine decarboxylase production	Positive
Catalase production	Positive
Glucose & Dulcitol fermentation	Positive
Reaction on triple sugar iron agar	Red slant, yellow butt and black precipitation with some H_2S production

The identity of *P. multocida* isolates was confirmed with the tests listed in Table 3, including Gram's stain. Additionally, the samples were enriched in Todd Hewitt broth (Oxoid, CM0189) for improved growth.

Table 3**: Tests used to identify *Pasteurella multocida***

Test	Result
Growth on selective media	Brain heart broth
Growth on McConkey agar	No growth
Haemolysis on blood agar	Negative
Oxidase production	Positive with exceptions
Catalase production	Positive
Glucose & sucrose fermentation	Positive
Dulcitol fermentation	Negative
Indole production	Positive with exceptions
Urease production	Negative
L-arabinose fermentation	Negative
D-sorbitol fermentation	Positive
D-xylose, Maltose fermentation	Variable
Nitrate production	Positive
Odour	Sweet

3.3 Antimicrobial Susceptibility Methods

The MIC and MPC were determined for all isolates in duplicate.

MIC procedure

The isolates were first tested using the broth microdilution method as recommended by the manufacturer (Sensititre plates, Trek Diagnostics, United Kingdom)(CLSI Document M31-A3, 2008). Commercial BOPOF and EQUI Sensititre MIC plates (Trek Diagnostics) were purchased for this purpose. Table 4 shows the different dilution ranges of the specific Sensititre plates. Each type of plate had a different set of antimicrobials and dilutions. The BOPOF plates for *P. multocida* required the addition of lysed horse blood. The EQUI plates were used for *S.* Typhimurium.

Table 4***:*** **Antimicrobial dilution ranges used on the specific microtitre plates**

Sensititre plate	Antimicrobial	Dilution Range (µg/ml)
BOPOF	Oxytetracycline	0.5 - 8
	Florfenicol	0.25-8
EQUINE	Enrofloxacin	0.25-2.0

MPC procedure

In this study two different methods were used to determine the MPC, namely the original method for MPC as described by Blondeau, (2009a) as well as an alternative modified method. The most important parameter for both methods was a final bacterial concentration of $\geq 10^9$ CFU/ml for each isolate.

A stock solution of the antimicrobial drugs was prepared: the type of antimicrobial determined the suspension solution. Both enrofloxacin and oxytetracycline dissolved easily in water, but florfenicol did not, so methanol was used as per certificate of analysis. Serial doubling dilutions of the stock solutions were made using the lowest MIC value obtained as the starting solution, e.g. 2-fold dilution, 4-fold dilution, 6-fold dilution, 8-fold dilution etc.

Stock solution: 0.25 g of the antimicrobial was added to 100 ml of sterile water/methanol and stored in a refrigerator.

Each working concentration was made up by adding different volumes of stock solution to the Mueller Hinton(MH) agar(Oxoid CM 0337). The three antimicrobials were purchased as powder: Sigma F1427 (Florfenicol), Sigma 04638 (Oxytetracycline), Fluka 17849 (Enrofloxacin) (please refer to Appendix A, B, C).

Table 5 defines the volume of antimicrobial stock solution used per working solution added to MH agar and the MPC method concentration.

Table 5**: Dilutions for stock solution added to working solution added to MH agar, to perform MPC method**

Amount of stock solution added (µg/ml)	**Concentration obtained (µg/ml)**
50	0.25
100	0.5
200	1
400	2
800	4
1.6	8
3.2	16
64	32

The procedure described by Blondeau (2009a) was used as follows:

The isolates were re-suspended and incubated for 24 hours at 37ºC. They were then plated out on 3-4 blood agar plates (90mm petri dishes) and incubated for 24 hours at 37 ºC, aerobically. After 24 hours the isolates were transferred into 100ml of Mueller Hinton broth and incubated overnight at 37ºC. The broth was centrifuged at 5000 rpm for 30 minutes, the supernatant discarded and the sediment re-suspended with 3 ml of fresh Mueller Hinton broth. One loop full of this suspension was then inoculated on previously prepared MH agar plates with different antimicrobial concentrations. The plates were incubated for 24 hours at 37 ºC, aerobically. The highest concentration, with no growth was regarded as the MPC value and expressed as µg/ml. Results were entered onto an EXCEL worksheet

Blondeau's method was followed for the *S.* Typhimurium isolates and an alternative method with the use of Todd Hewitt broth (Oxoid, CM 0189), instead of Mueller Hinton broth (Oxoid, 0337), for the *P. multocida.* The method was modified as follows:

The isolates were re-suspended and incubated for 24 hours at 37 ºC aerobically. The next day, each isolate was plated out on one blood agar plate and incubated 24 hours at 37ºC, aerobically. The growth was transferred to 30ml Todd Hewitt broth and incubated overnight at 37 ºC. The suspension was centrifuged at 5000 rpm for 30 minutes, discarding the supernatant. The sediment was re-suspended with 1 ml of Mueller Hinton broth and inoculated on previously prepared MH agar with different concentrations of antimicrobials. The concentration was measured against McFarland No.9 standard (Biomerieux, France), additionally with a spectrophotometer

(Densicheck, Biomerieux), to ensure the density is 10^9. Results are read as optical density and a McFarland standard). After 24 hours of incubation at 37ºC the plates were examined. The highest concentration, with no growth, was regarded as the MPC value. On each plate one quarter was left uninoculated where no antimicrobial was added, serving as a control for each plate. Figure 3 shows a plate divided into 4 quarters with the working concentration written in the middle. C indicated the control (no antimicrobial added), while the other quarters contained different isolates tested.

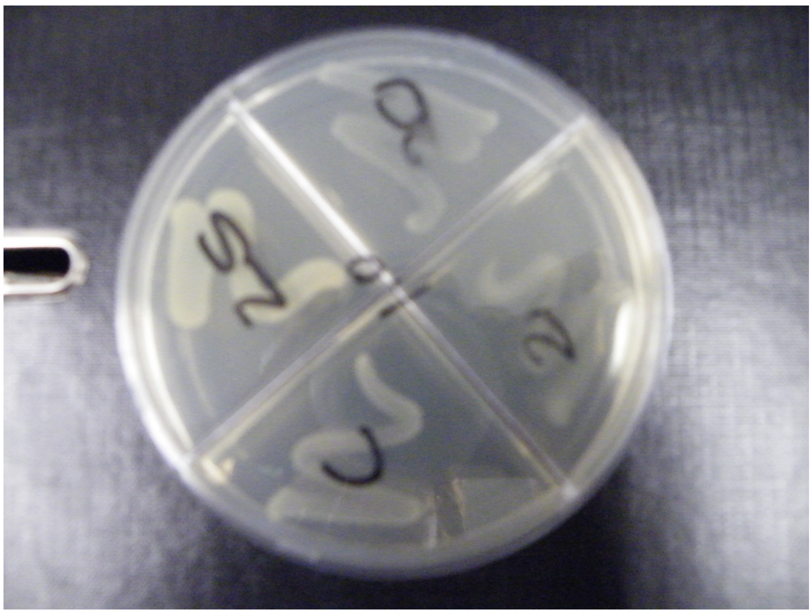

Figure 3: Photo of a modified Blondeau MPC method plate – to depict the numbering and concentration on the bottom of the plate

3.4 Calculations

Pharmacodynamic/Pharmacokinetic values were used as a measure to indicate bacterial inhibition and effective treatment with an antimicrobial.

The effectiv treatment with an antimicrobial was determined using the formula AUC/MIC, with a desired ratio of 125 to 250 h for optimal efficacy.

Bacterial inhibition by an antimicrobial was determined using the formula of C_{max}/MIC and AUC/MIC. The result of C_{max}/MIC must be between 8-12 to inhibit the organism, while an AUC/MIC must yield a result of ≥125 to minimize resistance.

CHAPTER 4

4. RESULTS

4.1 MIC & MPC values

The MIC values for enrofloxacin against 27 isolates of *S.* Typhimurium were all 0.25 µg/ml. The MPC values were all 0.5 µg/ml, except five strains with MPCs of 4 µg/ml. The MIC and MPC values for all 27 isolates are indicated in Appendix A.

Table 6*:* **Summary of MIC and MPC values for enrofloxacin against *Salmonella* Typhimurium**

MIC µg/ml	No. of samples	Percentage	MPC µg/ml	No. of samples	Percentage
0.25	27	100%	4	5	18.51%
			0.5	22	81.48%

The area highlighted in green represents the accepted range of the specific reference strains as per CLSI Document M31-A, vol.19. 2008, and the distribution of the 27 strains tested

Table 6 and Figure 4 depict the results obtained for the *S.* Typhimurium isolates included in the study. All the isolates had MICs of 0.25 µg/ml, while twenty two of the isolates had MPCs of 0.5 µg/ml. All the isolates had higher MPCs than MICs.

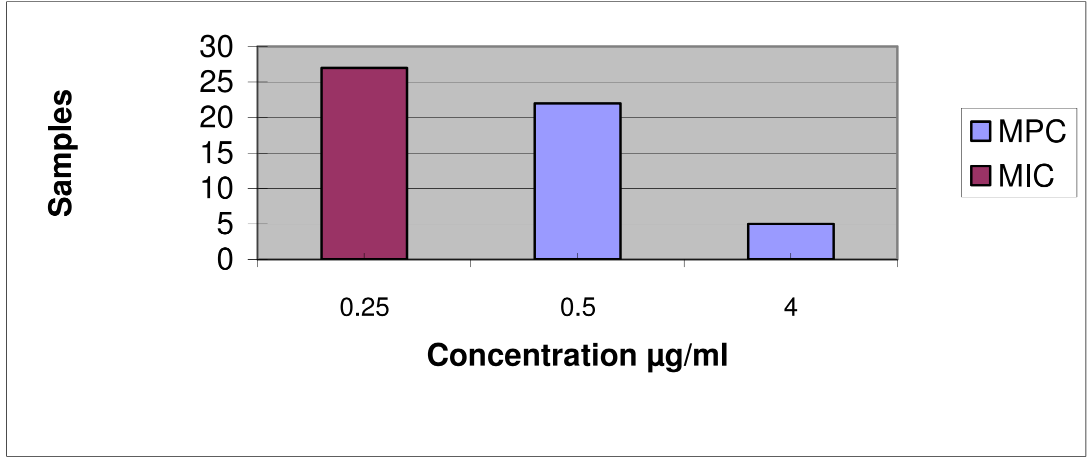

Figure 4: **Bar chart indicating the MIC and MPC values for enrofloxacin against 27 isolates of *Salmonella* Typhimurium**

Table 7**: Summary of MIC and MPC values for florfenicol against *Pasteurella multocida***

MIC µg/ml	No. of samples	Percentage	Susceptibility Interpretation	MPC µg/ml	No. of samples	Percentage	Susceptibility Interpretation
				32	3	10.34%	R
> 8			R	16	4	10.34%	R
8			R	8	4	13.79%	R
4			S	4	0		S
2	5	17.24%	S	2	2	3.45%	S
1	5	17.24%	S	<2	16	62.07%	S
0.5	8	27.59%	S	1			S
< 0.5	11	37.93%	S	<1			S

Key: R= Resistant, S= Sensitive. The area highlighted in red represents the isolates that is resistant against the antimicrobial tested as per of the specific reference strains as per CLSI Document M31-A2, 2008, and the distribution of the strains tested.

All the isolates of *Pasteurella multocida* strains yielded an MIC value that showed them to be sensitive to florfenicol. The MPC isolates yielded 18 isolates (65.52%) that were sensitive to florfenicol, while 11 isolates (34.48%) yielded MPC values that were resistant to florfenicol. All the isolates had a higher MPC than MIC value. Six of the isolates had an MIC/MPC ratio that was either the same or varied only by one dilution (refer to Table 4, Figure 3 and Appendix B). Most of these isolates were obtained from samples collected as part of a routine survey of cattle for resistance to antimicrobial drugs.

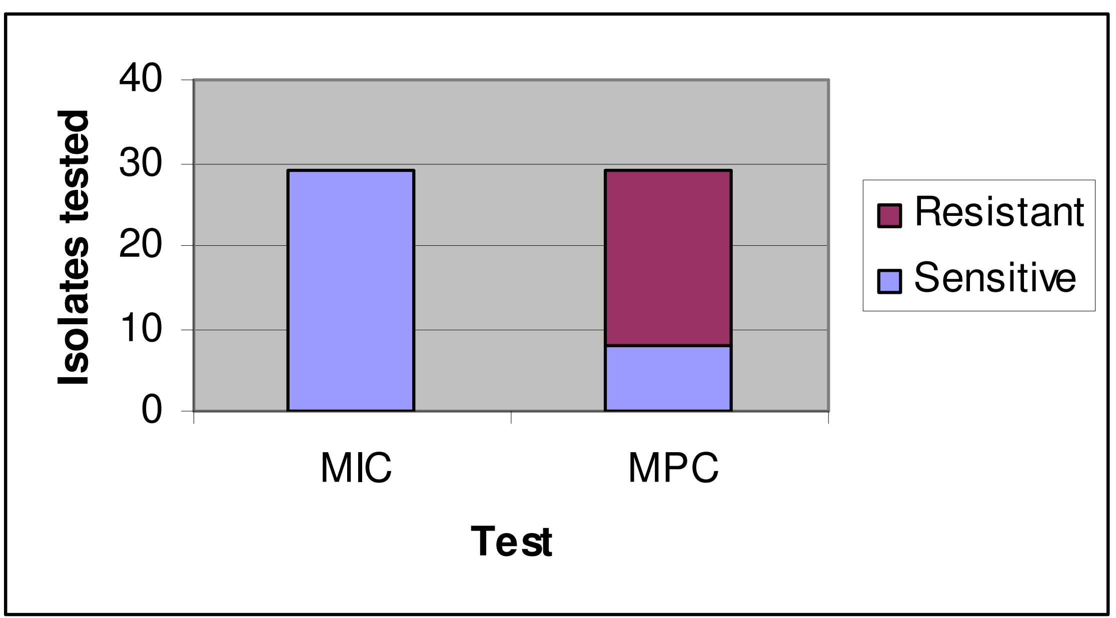

Figure 5**: Comparative MIC and MPC values for florfenicol against *Pasteurella multocida***

Table 8: **Summary of MIC and MPC values for oxytetracycline against *Pasteurella multocida***

MIC µg/ml	No. samples	Percentage	Susceptibility Interpretation	MPC µg/ml	No. samples	Percentage	Susceptibility Interpretation
>8	6	20.69%	I	16	16	55.17%	R
8	6	20.69%	I	8	1	3.45%	I
4	1	3.45%	S	4	5	17.24%	S
2	4	13.79%	S	2	1	3.45%	S
1	5	17.24%	S	1	5	17.24%	S
0.5	7	24.14%	S	≤1	1	3.45%	S

Key: R= Resistant, S= Sensitive. The area highlighted in red represents the isolates that is resistant against the antimicrobial tested as per of the specific reference strains as per CLSI Document M31-A2, 2008 , and the distribution of the strains tested.

Seventeen isolates (58.62%) of *Pasteurella multocida* yielded a susceptible MIC value and twelve isolates (41.38%) had an intermediate value. The MPC testing indicated that twelve isolates had a susceptible MIC value, while only one was intermediate. Sixteen of the isolates (55.17%) yielded an MPC value that showed them to be resistant to oxytetracycline (refer to Table 5, Figure 4 and Appendix C). Five of the isolates had an MIC/MPC ratio of 0.

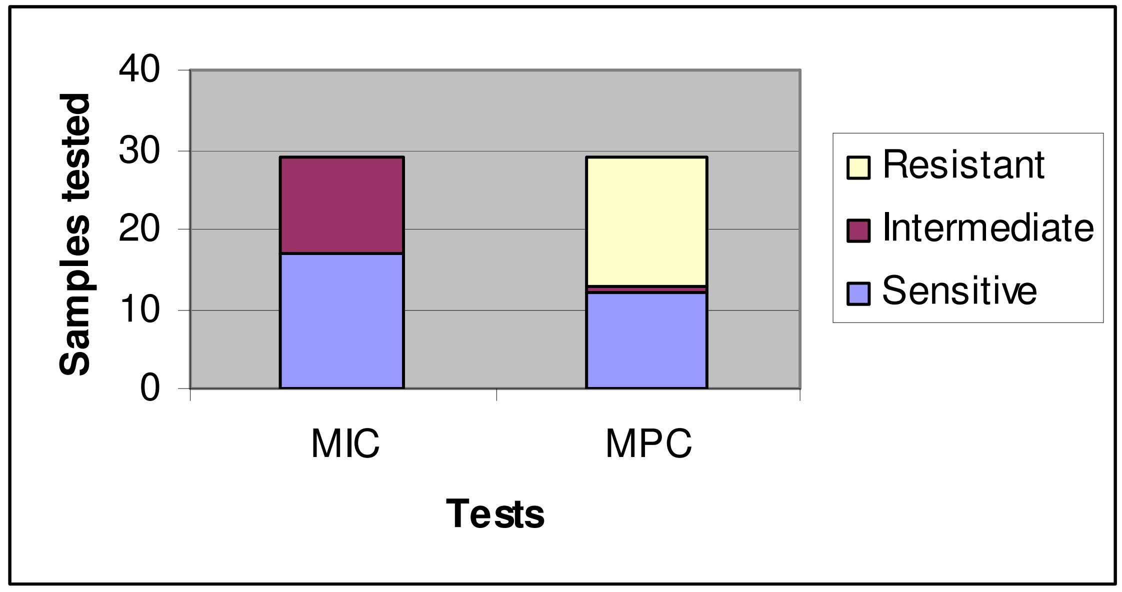

Figure 6: **MIC and MPC values for oxytetracycline against *P. multocida***

4.2 Calculation of MIC and MPC ratios and PK/PD parameters

The calculation of the MIC and MPC ratios was performed to determine how much the MIC and MPC values differed for each bacterial strain tested, and both the MIC and MPC $_{50}$ and $_{90}$ ratios were calculated. The ratios in comparison to a value of 1 are indicated in Figures 7 and 8. The closer the MIC and MPC values, the more effective the antimicrobial action will be.

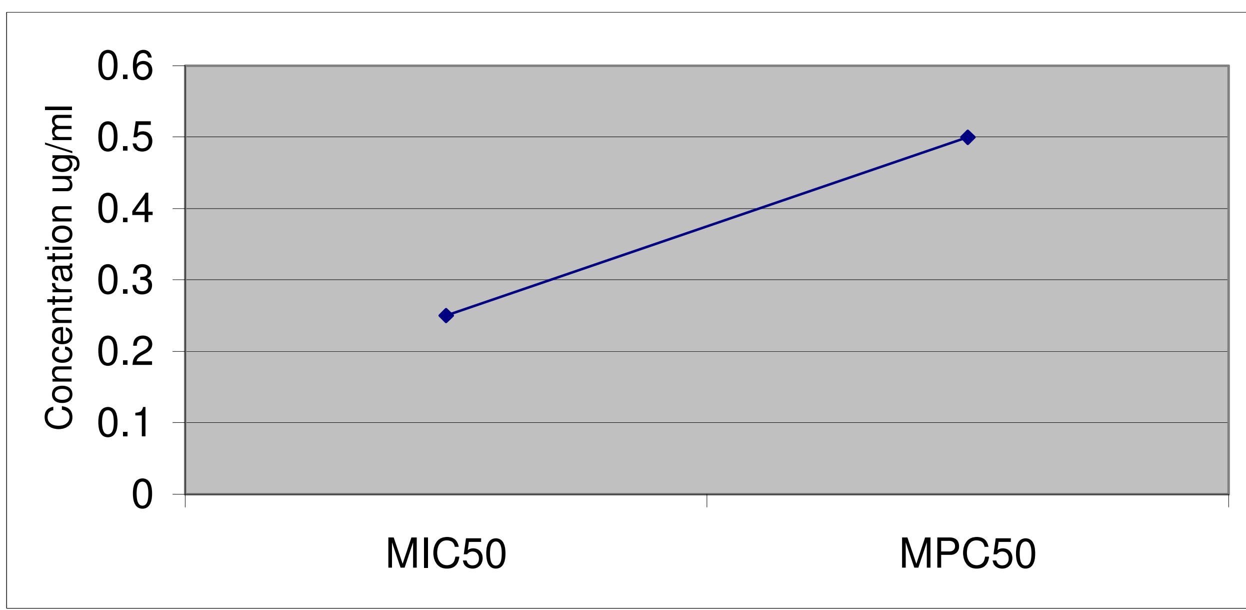

Figure 7: **MIC_{50}:MPC_{50} ratio for enrofloxacin against *Salmonella* Typhimurium yielded a value of 2**

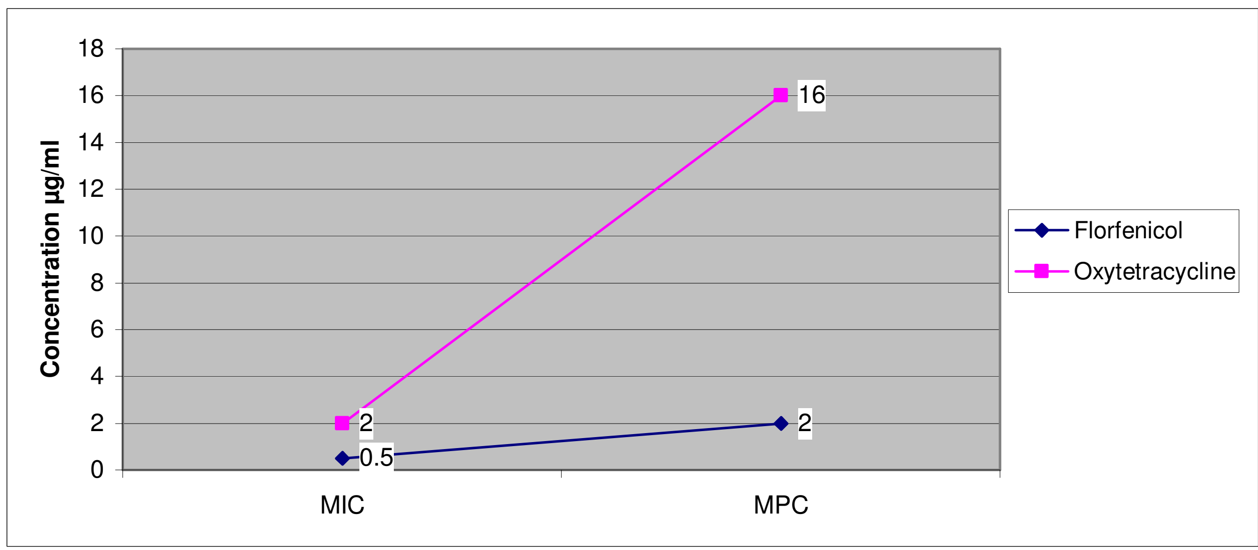

Figure 8**: Both MIC_{50}:MPC_{50} ratios for oxytetracycline and florfenicol against *P. multocida* yielded values respectively of 4 for oxytetracycline and almost 4(0.5:<2) for florfenicol.**

Table 9 provides the summary of all the results obtained in the study for the isolated organisms against the applicable antimicrobial drugs. This summary indicates the differences between the MIC and MPC values.

Table 9**: Summary of results for all 3 organisms**

Antimicrobial	Organism	No. of samples tested	MIC_{50} µg/ml	MPC_{50} µg/ml	MIC_{50}:MPC_{50} ratio	MIC_{90} µg/ml	MPC_{90} µg/ml	MIC_{90}:MPC_{90} ratio
Enrofloxacin	*Salmonella* Typhimurium	27	0.25#	0.5	0.25#:0.5	0.25#	4	0.25#:4
Florfenicol	*Pasteurella multocida*	29	0.5	<2	0.5:<2	2	>32	2:>32
Oxytetracycline	*Pasteurella multocida*	29	2	16	2:16	>8	16*	>8:16*

*50-100% of the isolates yielded an MPC value of >16 µg/ml

100% of the isolates yielded an MIC value of 0.25 µg/ml

The PD/PK parameters were used in conjunction with the MIC and MPC values to determine the antimicrobial's efficacy against the specific organism in terms of a favourable clinical response and minimization of antimicrobial resistance selection. Table 9 in conjunction with Table 10 was used to determine the efficacy of the antimicrobials in this study. Criteria in Table 11 were used to determine the efficacy of the antimicrobials to inhibit the growth of the organisms. The C_{max}, T and AUC values were obtained from previous documented studies.

Table 10**: Summary of pharmacodynamic/pharmacokinetic data for the results obtained**

Antimicrobial	Organism	PD/PK parameter to determine efficacy	
		Calculation	Standard measure for efficacy
		AUC/MIC	AUC/MIC = 125 to 250 for optimal efficacy
Enrofloxacin	*S.* Typhimurium	Not done – Extra-label use	
Florfenicol	*P. multocida*	#283.56	
Oxytetracycline	*P. multocida*	*56	

^C_{max} plasma concentration: ^ Giguere *et al.*, 2011. # Concentration of C_{max} and AUC: Schering plough, 2008. $Hesje *et al.*, 2007

Table 11: **Summary of pharmacodynamic/pharmacokinetic data for the results obtained**

Antimicrobial	Organism	PD/PK parameter to determine bacterial inhibition	
		Calculation	**Standard measure**
		C_{max}/MIC ratio	C_{max}/ [$]MIC = 8-12 to minimize resistance
Florfenicol	*P. multocida*	[#] 9.38	
Oxytetracycline	*P. multocida*	^2.58	

^C_{max} plasma concentration: ^ Giguere *et al*., 2011 [#] Concentration of C_{max} and AUC Schering plough.2008 [$]Hesje *et al*.,2007

CHAPTER 5

5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Antimicrobial resistance testing has been done for all antimicrobials and the information gained from these studies contributed to the successful treatment of patients. The reason for many of the flawed MIC and MPC clinical studies is that patients infected with resistant pathogens are often not included in the studies (Blondeau, Hansen, Metzler & Hedlin, 2004). The studies are limited to testing only one dose; endpoint measurements are incorrectly defined; and due to the high specificity of the inclusion criteria, the studies are not reflective of the true situation in the field. For this study, isolates from surveillance programmes (44.82%) and clinical cases (55.17%) were included for the testing of florfenicol and oxytetracycline against *P. multocida*, while all *S.* Typhimurium isolates were obtained from clinical cases.

In published literature, two distinct opinions with regard to MIC and MPC testing exist. Researchers prefer either the one or the other method. There seems to be no documented study that recommends the use of both MPC and MIC testing (Blondeau *et al.,* 2007a). Both methods have a place in susceptibility testing. However, the MIC can be used daily for most organisms isolated in diagnostic laboratories, while the MPC is currently used for infections that are difficult to treat and for research purposes.

The MIC procedure used in this project was described by the CLSI and the results were read according to its standard M31-28 (2008). The MIC method is usually performed with a bacterial concentration of approximately 10^5 CFU/ml or >100 colonies per plate. The MIC method may be influenced by the incubation period, incubation temperature and the media/broth used (Blondeau, 2009a).

In the case of fastidious organisms such as *P. multocida*, it is recommended that use is made off a selective medium such as Todd Hewitt or *Haemophilus* test medium, which will enrich the growth of the organisms on the primary plates. Initially in this study, the MIC method did not yield satisfactory results for *P. multocida* when using the method as recommended by the manufacturer. Following a query, the manufacturer suggested the addition of lysed horse blood to the MH broth before adding the inoculum to the 96-well plates (Trek). This improvement made the reading of the results much easier, as bacterial growth was clearer. During the reading of MIC results, some problems may arise such as fading end-points (where end-points are not distinct) or skips (a well with no growth, bordered by two wells with growth). The fading end-points were limited in this study and methods were repeated if either skips or fading end-points were encountered. The factors that influenced the results most were differences with regard to the inocula size and the incubation time. It is recommended that trial runs are conducted before implementing commercial MIC methods in a diagnostic laboratory.

The clinical breakpoint of the CLSI guideline incorporates both the pharmacodynamic and pharmacokinetic attributes of the isolates (Boothe, 2006). The clinical breakpoint is useful as a tool for clinical infections but has no epidemiological significance. Results are interpreted as sensitive, intermediate or resistant (Silley, Bywater & Simjee, 2006). Furthermore, the MIC values are obtained from *in vitro* bacterial growth and within a clinical reference range, it will indicate a possible response *in vivo*.

The susceptibility breakpoint of enrofloxacin for animal pathogens is ≤0.5 µg/ml and the resistant breakpoint is ≥4 µg/ml. Therefore in this study no MIC values pointed to resistance. The susceptibility breakpoint of florfenicol for animal pathogens is ≤2 µg/ml, and the resistant breakpoint is ≥8 µg/ml. No MIC values pointed to resistance The resistant breakpoint of oxytetracycline for animal pathogens is ≥16 µg/ml, and the susceptibility breakpoint is ≤4 µg/ml (Booth, 2006). Twelve of the MIC values were intermediate and the rest were in the susceptible range. These breakpoints were used as the reference range in this study. The best MIC value that can be obtained with testing for treatment will be the opposite of the resistant breakpoint. The nearer the value to the resistant breakpoint, the higher is the chance that treatment can contribute to the development of resistance to the specific antimicrobial.

The clinical reference range was used as an indicator of antimicrobial drug susceptibility however, because enrofloxacin is not registered in South Africa for use in horses, the general clinical reference range was used for *S*. Typhimirium isolates. Some animal species still lack official clinical breakpoints, and human breakpoints are often used as guidelines, the reason being that the antimicrobial has not been registered for animal use Or that the NCLLS has not yet determined species-specific breakpoints for specific antimicrobials. The clinical reference range of enrofloxacin is 0.5 to 4 µg/ml (CLSI, 2008). The MIC_{50} value of enrofloxacin for *S.*Typhimurium was 0.25 µg/ml. Unfortunately there are no official values to measure it against. This indicates that treatment of horses with enrofloxacin was likely adequate when the drug was used by veterinarians extra-labelly. The limited results point to the fact that the use of enrofloxacin has thus far not been abused in the equine industry. The MIC_{90} concentration of enrofloxacin for *S.*Typhimurium was also 0.25 µg/ml.

The clinical reference range for florfenicol against *P. multocida* infections is 2 to 8 µg/ml. Eleven isolates had MIC values below the MIC_{50}. The MIC of florfenicol for *P. multocida* was within the range when using either the MIC_{50} (0.5 µg/ml) or MIC_{90} (<2 µg/ml) as calculated in this study. This shows that the treatment of animals with standard doses of florfenicol suffering from infections with these isolates will be well within the reference range of the antimicrobial. During this study the mean MIC concentration of florfenicol for *P. multocida* was higher at 0.5 µg/ml, while another study found the MIC values for *P. multocida* 0.47 µg/ml for cattle and 0.51 µg/ml for pig strains (Hörmansdorfer, 1998). In a study by Sweeney, Brumbaugh and Watts (2008), 10 *P. multocida* isolates had a MIC_{50} value of 2 µg/ml and an MIC_{90} of 4 µg/ml for florfenicol, while the MIC_{50} for oxytetracycline was 0.25 µg/ml and the MIC_{90} 32 µg/ml.

The clinical reference range of oxytetracycline for *P. multocida* is 4-16 µg/ml**.** Treatment according to the results of this study will therefore fall within the clinical reference range as described by the CLSI for MIC_{50} values. With an MIC_{50} value of 2 µg/ml it is below the clinical reference range of the antimicrobial. Sixteen isolates (55.17% of the samples) had MIC values below the clinical breakpoint. This indicated that in these isolates, no resistance was present. One of the isolates had an MIC value of 4 µg/ml, which is the lower value of the reference range. For 6 of the isolates tested (20.69% of the samples), the MIC_{90} was 8 µg/ml, while 6 isolates had an MIC >8 µg/ml. This is not surprising when considering the fact that oxytetracycline is the most used (and abused) antimicrobial drug used in cattle in South Africa.

Where the MIC_{90} is above the resistant clinical breakpoint the treatment will usually be unsuccessful. During a study by Giguere *et al.,* (2011) with bovine respiratory disease-causing organisms in cattle, the MIC_{90} values of florfenicol and oxytetracycline against *P. multocida,* were 0.5 µg/ml and 1 µg/ml respectively. The MIC_{90} values in this study were 2 µg/ml and >8 µg/ml respectively. Both these values are much higher than the reference range as per CLSI.

The area below the MIC value indicates that the specific organisms will be treated effectively with the prescribed dosages. Eleven of the *P. multocida* isolates fell below the MIC_{50} value of florfenicol for this study. Twelve isolates of *P. multocida,* fell below the MIC_{50} value of oxytetracycline for this study.

The modified and original MPC method gave similar results for the *P. multocida* isolates against the antimicrobials florfenicol and oxytetracycline. The two methods differed only in the sense that a 90mm petri dish, divided into quarters was used as an alternative to the whole plate per isolate and that the volumes of reagents used were less. Thereby better utilization was made of the media available in the laboratory. It also saved space in the incubators and on reagent volumes.

The *S.* Typhimurium isolates had a low MPC_{50} value for enrofloxacin. The use of enrofloxacin is common practice in horses, despite the fact that in South Africa it is not a registered antimicrobial drug for use in horses. Veterinary practitioners usually treat a horse using the same dose as for cattle (Boeckh, Buchanan, Boeckh, Wilkie, Davis, Buchanan & Boothe, 2001). The results obtained from the *S.* Typhimurium isolates confirmed the results of previous studies. The MPC_{50} values showed a four-fold increase from the MIC_{50}. The MPC_{90} concentration for this study was 4 µg/ml, thus a 16-fold increase from the $MIC_{90.}$

The MPC_{50} results obtained for florfenicol against *P. multocida* were <2 µg/ml for 16 (62.07% of the tested) isolates. This MPC value is still below the clinical breakpoint for florfenicol. The clinical reference range represents an MPC_{50} of 2 µg/ml and an MPC_{90} of >32 µg/ml. The MPC_{90} concentrations of this study fall outside the clinical reference range. The MPC_{90} concentration represents an alternative to the MIC_{50} values in this study and using higher dosages to exceed the MPC_{90} will theoretically be a more effective treatment regimen to

minimize resistance development. However, higher concentrations of the drug for treatment must first be tested for safety before using it as a treatment regimen as it may be toxic for several vital organs.

The results for oxytetracycline against *P. multocida* showed an MPC_{50} value of 16 µg/ml for 16 (55, 17% of the tested) isolates. Treating animals to reach an MPC_{50} value 16 µg/ml will be within the clinical reference range of the organism. In this study, both MPC_{50} and MPC_{90} values were 16 µg/ml. This creates the need for susceptibility methods such as MPC, which can determine drug concentrations that will kill first step mutants. However, the safety of this concentration should likewise be determined first, before using it for therapy.

The mutant selection window shows the correlation between the MIC_{50} and MPC_{50} values as well as the effectiveness of the treatment/dosing. This is the concentration where the selective amplification of the organism occurs and where resistant populations can develop. Time-dependent antimicrobials that stay within the mutant selection window such as oxytetracycline promote the chances of resistance. Twenty-two (81.48%) of the *S.* Typhimirium isolates treated with enrofloxacin yielded results similar to the MIC_{50} and MPC_{50} values. *P. multocida* had 2 isolates similar to the MIC_{50} and MPC_{50} values for oxytetracycline, and only one isolate had MIC and MPC values within the mutant selection window. None of the *P. multocida* isolates exposed to florfenicol fell between the MIC_{50} and MPC_{50} values. The isolates with concentrations at the MPC value will block first-step mutation.

The closer the MIC:MPC ratio is to each other the higher the suitability of the antimicrobial (Zhao & Drlica, 2001). The MIC_{50} and MPC_{50} ratios for enrofloxacin against *S*. Typhimirium was 0.25:<0.50 and 0.5:<2 for florfenicol against P multocida, indicating that current dosages used will be suitable for treatment. However, the MIC_{50}:MPC_{50} ratio of 2:>16 for oxytetracycline against *P. multocida* indicates that treatment at much higher dosages may be indicated but might lead to toxicity at this concentration. The MIC_{50}:MPC_{50} ratio in this study is similar to the clinical reference range for oxytetracycline. The MPC values were higher than the MIC values as was expected.

The MIC_{90}:MPC_{90} ratio for enrofloxacin against *S.* Typhimirium was <0.25:4, therefore a 16-fold difference. The MIC_{90}/MPC_{90} ratio for florfenicol against *P. multocida* was 2:>32, a 16-fold difference and the MIC_{90}:MPC_{90} ratio of >8:6 for oxytetracycline represents a 2-fold difference.

MPC values above the MPC_{50} will block both susceptible and mutant bacterial growth however, this can be an indication of second step mutations. It is important to know that the MPC will block only the least susceptible bacteria and that it is independent of the mechanism of resistance. Among the *S.* Typhimurium isolates were 5 strains with MPC values above the MPC_{50} value. There were no *P. multocida* isolates exposed to oxytetracycline with MPC values above the MPC_{50}. There were eleven isolates of P. *multocida* exposed to florfenicol with MPC values above the MPC_{50} value. The mutant selection window can therefore be determined and indicate if a

positive clinical response, without selecting for resistance, is possible. The pharmacokinetic/pharmacodynamic parameters can be calculated with the aid of MIC and MPC values. The MPC values in the pharmacodynamic/pharmacokinetic parameter calculation are unknown and this still needs further research.

It must be kept in mind that the PK/PD parameters such as AUC (a measure of the total amount of antimicrobial drug present over a defined time period), T>MIC and C_{max} all depend on the dose and the infection site. For this study these values were obtained from previously documented studies. It supports the results of the MIC and MPC test results of this study. Published literature indicates that C_{max}/MIC must be 8-12, to be clinically effective and to reduce development of resistance. The AUC/MIC should be >125 to have a positive clinical response and minimization of antimicrobial resistance development. The AUC/MPC_{50} calculation with a result of ≥22 for Gram-negative organisms can reduce the possibility of resistance, (Hesje *et al.,* 2007). Unfortunately because enrofloxacin is not registered for use in horses, the PK/PD parameters cannot be calculated. The response of *P. multocida* isolates to florfenicol measured with the PD/PK parameters gave the following results: AUC/MIC value of 283.56 and an AUC/MIC_{90} value of 70.89 indicative that the treatment will be effective with a positive clinical response. The AUC/MIC value of 56 for *P. multocida* isolates exposed to oxytetracycline indicated that the treatment will be unsuccessful. The PK/PD parameter C_{max}/MIC indicated that florfenicol at the MIC_{50} will minimise resistance with a value of 9.38, and oxytetracycline at the MIC_{50} concentration will not prevent the occurrence of resistance in the P. *multocida* organisms tested in this study with a value of 2.85.

5.2 Conclusion and recommendations

Distinction was made between the MIC_{50}, MIC_{90} and the MPC_{50}, MPC_{90} because this indicates either the value where the antimicrobial will be effective against 50% of the isolates or against the majority (90%) of the isolates. This information will be useful in treatment of highly resistant bacteria.

Enrofloxacin is not registered in South Africa for use in horses. The dose of enrofloxacin for cattle is applied for the treatment of horses at 7.5mg/kg for Baytril 100® (IDR, 2005/6). It is advisable that the pharmaceutical industry obtain registration of enrofloxacin for use in horses to ensure the use of the correct dose and to prevent development of resistance to the drug. This will prevent off-label use and preserve the antimicrobial for the future.

If a practitioner interprets MIC values correctly, a less toxic and cheaper dosing strategy can be used when treating patients as the spread of susceptible bacteria will be prevented. MIC results can be seen as the reference point but when treating a patient based on MIC results, it will not prevent the growth of resistant mutants. The MIC value will be the best parameter for most clinical cases. Failure of treatment based on MIC results might reduce the occurrence of the resistant organisms, but it will also enhance the risk of failure in effectively treating the pathogen.

The MPC method is more expensive and there is a risk of toxicity and other adverse effects at the higher dosing strategy. By basing dosing strategies on MPC test results, the occurrence of first step mutants will be prevented and the growth of resistant bacteria will be inhibited. The results will indicate the usage of a dosage against bacterial infections at a bacterial concentration of 10^9 (the concentration at which the bacteria are likely to occur during an infection). By basing treatment on the MPC value, it will result in the use of higher concentrations of the antimicrobial over a shorter time period, thus spending a shorter time in the mutant selection window. This strategy offers clinical efficacy with a minimal exposure time to antimicrobial drugs. Mutant prevention concentrations are obtained in a minimum of time for the target pathogens.

Even though the MPC values can be the applicable solution to the successful treatment of sick animals, the pharmaceutical industry should first determine the safety of the antimicrobials at higher dose rates or higher active ingredient concentrations as well as the safety of the patient when treated at shorter intervals, but at the higher concentration. Only then can practitioners start using the MPC results for the treatment of clinical cases. It should be borne in mind that when organisms are resistant to the MIC value, the MPC value will not be effective either. This was confirmed by studies where the mechanism of resistance of quinolones has been identified as mutations in genes that encode for DNA gyrase. The CLSI guidelines exclude any isolate being tested for MPC, if resistant to MIC.

MIC and MPC results should be linked to the *in vivo* plasma pharmacokinetic parameters for a particular organism. PK-PD modeling is a specific science that has been designed to optimize the dosage regimen of antimicrobial drugs in times of every increasing resistance. Therefore, by analyzing the PD/PK in conjunction with MIC/MPC test results, it will prolong the life and efficacy of an antimicrobial. No *in vitro* tests, such as MIC and MPC methods can account for the patient's immune response and antimicrobials can only work alongside the patient's immune response or natural defense mechanisms. In animals, the housing, interaction with other animals and climate play additional roles by creating stress factors. Preventative treatment of animals against diseases such as pneumonia might also be the reason for resistant mutations occurring.

A dosing strategy must be effective in eradicating the microbial infection and minimize the occurrence of resistance. The best treatments are possible only if the practitioner has meaningful information available. The decision about the optimal antimicrobial to be used and the dose can only be made if the practitioner has a full history of the animal as well as culture and sensitivity results from the laboratory.

Another possibility of preventing the formation of mutants and resistant bacteria against antimicrobials is to use combination antimicrobials and not single antimicrobials. This might be the safer option with regard to avoidance of toxicity in the recipient animal. However, the MIC and MPC values should be known before using the antimicrobials in combinations. Combinations should be carefully selected and their bacterial action should be considered before administration.

In conclusion, the goal with antibiotic susceptibility testing is to achieve two objectives. The first is to apply effective antimicrobial treatment, and this can be achieved by means of MIC determinations. The second is to minimise the occurrence of bacterial resistance. The MPC method is one more tool that can be applied to strive for better control of the development of resistance to antimicrobial drugs.

REFERENCES

AARESTRUP, F.M. 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *International Journal of Antimicrobial Agents*, 12: 279-285.

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

BLONDEAU, J.M. 2009b. *STAT companion animals: steps to antimicrobial therapy*. Northern Compendiums Inc. Canada.

BLONDEAU, J.M. 2012. Challenges and new developments in canine pyoderma. The Three M's. *Today's Veterinary Practice.* Jan/Feb:32-33.

BLONDEAU, J.M., XILIN, Z., HANSEN, G. & DRLICA, K. 2001. Mutant prevention concentrations of flouroquinolines for clinical isolates of *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotheraphy*, 45: 433-438.

BLONDEAU, J.M. HANSEN, G, METZLER, K & HEDLIN, P. 2004. The role of PK/PD parameters to avoid selection and increase of resistance: Mutant prevention concentration. *Journal of Chemotherapy*, 16(3): 1-19.

Blondeau, JM., Borsos, SD., Blondeau, LD., Blondeau, BJ. and Hesje, CH. 2007a. Comparative minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC) values of enrofloxacin, florfenicol, tilimicosin, tulathromycin against *Mannheimia haemolytica* (MH) isolates from cattle with bovine respiratory disease. *International Meeting on Emerging Diseases and Surveillance (IMED). Vienna, Austria, 23-25 February 2007.*

Blondeau, JM., Borsos, SD, Blondeau, LD., Blondeau, BJ. & Hesje, CH. 2007b. Concentration dependent killing of *Mannheimia haemolytica* (MH) by enrofloxacin at the minimum inhibitory, mutant prevention, maximum serum and tissue drug concentrations *International Meeting on Emerging Diseases and Surveillance (IMED). Vienna, Austria, 23-25 February 2007.*

BLONDEAU, J.M., BORSOS, S., BLONDEAU, B.J.J. & HESJE, C.H. 2012. Comparative minimum inhibitory and mutant prevention drug concentrations of enrofloxacin, ceftiofur, florfenicol, tilmicosin and tulathromycin against bovine clinical isolates of *Mannheimia heamolytica*. *Veterinary Microbiology*, 160: 85-90.

BOECKH, C., BUCHANAN, C., BOECKH, A., WILKIE, S., DAVIS, C., BUCHANAN, T. & BOOTHE, D. 2001. Pharmacokinetics of the Bovine Formulation of Enrofloxacin (Baytril® 100) in Horses. *Veterinary Therapeutics,* 2: 129-134.

BOOTHE, D.M. 2006. Principles of antimicrobial therapy. *Veterinary Clinics: Small Animal Practice*. 36: 1003-1047.

BURCH, D.G.S. 2007. *The rational use of antimicrobials in relation to antimicrobial resistance*. Old Windsor, Berkshire, United Kingdom.
http://www.octagon-services.co.uk/articles/antibioticsEnglish.pdf

BYARUGABA, D.K. 2004. A view on antimicrobial resistance in developing countries and responsible risk factors. *International Journal of Antimicrobial Agents,* 24: 105-110.

CAPRIOLI, A., BUSANI, L., MARTEL, J.L. & HELMUTH, R. 2000. Monitoring of antimicrobial resistance in bacteria of animal origin: epidemiological and microbiological methodologies. *International Journal of Antimicrobial Agents*. 14: 295-301.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI). 2008. *Performance standards for antimicrobial disk and dilution susceptibility methods for bacteria isolated from animals; approved standard – second edition*. CLSI document M31-A2. Philadelphia: CLSI.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI). 2008. *Performance standards for antimicrobial disk and dilution susceptibility methods for bacteria isolated from animals; approved standard – third edition*. CLSI document M31-A3. Philadelphia: CLSI.

Antimicrobial breakpoint estimation accounting for variability in pharmacokinetic. *Theoretical Biology and Medical Modelling*, 6: 10.

DRLICA, K. 2003. The mutant selection window and antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*, 52: 11-17.

DRLICA, K., ZHAO, X., BLONDEAU, J.M., & HESJE, C. 2006. Low correlation between MIC and mutant prevention concentration. *Antimicrobial Agents and Chemotherapy*, 50: 403-404.

Food and Agricultural Organization/World Health Organization/World organization for Animal Health(OIE). 2008. Joint Food and Agricultural Organization/World Health Organization/World organization for Animal Health Expert Meeting on Critically Important Antimicrobials. Report of a meeting held at the Food and Agricultural Organization, Rome, Italy, 26–30 November 2007.

GIGUERE, S. & TESSMAN, R.T. 2011. Rational dosing of antimicrobial agents for bovine respiratory disease: The use of plasma versus tissue concentrations in predicting efficacy. *Journal of Applied Research in Veterinary Medicine.* 9: (4).

GNANOU, J.C. & SANDERS, P. 2000. Commentary, Antimicrobial resistance in bacteria of animal origin: Methods in use to monitor resistance in European Union countries. *International Journal of Antimicrobial Agents*. 15: 311-322.

HESJE, C.E., TILLOTSON, G.S. & BLONDEAU, J.M. 2007. MICs, MPCs and PK/PDs: a match (sometimes) made in hosts. *Expert Review of Respiratory Medicine*, 1: 7-16.

HENDRIKSEN, R.S., MEVIUS, D.J., SCHROETER, A., TEALE, C., MEUNIER, D., BUTAYE, P., FRANCO, A., UTINANE, A., AMANDO, A., MORENO, M., GREKO, C., STARK, K., BERGHOLD, C., MYLLYNIEMI, A.L., WASYL, D., SUNDE, M. & AARESTRUP, F.M. 2008. Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European Countries: 2002-2004. *Acta Veterinaria Scandinavica*, 50(28): 1-10.

HÖRMANSDORFER, S. & BAUER, J. 1998. Resistance of bovine and porcine Pasteurella to florfenicol and other antimicrobials. *Berl Munch Tierarztl Wochenschr.* 111: 422-6.

Swan, G. 2005. IDR.2005/2006. 2005. IVS Desk reference Editor: G.Swan. Volume 8. Johnic Publishing.CTP book Printers, Cape

LANGSTON, V.C, SEDRISH, S. & BOOTHE, D.M. 1996. Disposition of single–dose oral enrofloxacin in the horse. *Journal of Veterinary Pharmacology and Therapeutics.*19: 316-319.

LOVEMORE, D.M. 2005. Minimizing antimicrobial resistance. Personal view. *Lancet Infectious Diseases.* 5: 450-59.

MARTINEZ, M. & SILLEY, P. 201O. Antimicrobial drug resistance, IN; Comparative and Veterinary pharmacology 199. Handbook of experimental Pharmacology. Springer- verlag Berlin, Heidelberg

METZLER, K., HANSEN, G.M., HEDLIN, P., HARDING, E., DRLICA, K. & BLONDEAU, J.M. 2004. Comparison of minimal inhibitory and mutant prevention drug concentrations of 4 fluoroquinolones against clinical isolates of methicillin-susceptible and -resistant *Staphylococcus aureus*. *International Journal of Antimicrobial Agents,* 24: 161–167.

SCHERING PLOUGH ANIMAL HEALTH. 2008. NADA, 141-265. Nuflor gold injectable solution florfenicol, for beef and dairy cattle.

OKEKE, I.N., KLUGMAN, K.P., BHUTTO, Z.A., DUSE, A.G., JENKINS, P, O'BRIEN, T.F., PABLOS- MENDEZ, A. & LAZMINARAYAN, R. 2005. Antimicrobial resistance in developing countries. Part II: Strategies for containment. *Lancet Infectious Diseases.* 5: 568-80.

PASQUALI, F. & MANFREDA, G. 2007. Mutant prevention concentration of ciprofloxacin and enrofloxacin against *Escherichia coli*, *Salmonella* Typhimurium and *Pseudomonas auerignosa*. *Veterinary Microbiology*. 119: 304-310.

PRIEBE, S. & SCHWARZ, S. 2003. In vitro activities of florfenicol against bovine and porcine respiratory tract pathogens. *Antimicrobial Agents and Chemotherapy*. 47(8): 2703 -2705.

Quinn, P.J., Carter, M.E., Markey, B. & Carter, G.R. 1994. *Clinical Veterinary Microbiology.* London: Wolfe Publishing.

RERAT, M., ALBINI, S., JAQUIER, V. & HUSSY, D. 2012. Bovine respiratory disease: Efficacy of different prophylactic treatments in veal calves and antimicrobial resistance of isolated Pasteurellaceae. *Preventative Veterinary Medicine* 103: 265-273.

SCHWARZ, S., KEHRENBERG, C. & WALSH, T.R. 2001. Use of antimicrobial agents in veterinary medicine and food animal production. *International Journal of Antimicrobial Agents*, 17: 431-437.

SILLEY, P., BYWATER, R. & SIMJEE, S. 2006, J07 Antimicrobial breakpoints - an area for clarification. *Journal of Veterinary Pharmacology and Therapeutics*, 29: 310–311.

SMITH, H.J., NICHOL, K.A., HOBAN, D.J. & ZHANEL, G.G. 2003. Stretching the mutant prevention concentration (MPC) beyond its limits. *Journal of Antimicrobial Chemotherapy.* 51: 1323–1325.

Songer, J.G. & Post, K.W. 2005. *Veterinary Microbiology.* Bacterial and fungal agents of animal disease. Elsevier Inc. China:

SWEENEY, M.T., BRUMBAUGH, G.W. & WATTS, J.L. 2008. In vitro activities of tulathromycin and ceftiofur combined with other antimicrobial agents using bovine *Pasteurella multocida* and *Mannheimia haemolytica* isolates. *Veterinary Therapeutics*. 9: 212-222.

TEUBER, M. 2001. Veterinary use and antibiotic resistance. *Current Opinion in Microbiology.* 4: 493-499.

TREK DIAGNOSTIC SYSTEMS. 2005. Sensititre 18-24 hour MIC and breakpoint susceptibility plates and just one strips. For veterinary use. www.trekds.co./techinfo.

WATTS, J.L., YANCEY, R.J.(JR)., SALMON, S.A. & CASE., C.A. 1994. A 4-year survey of antimicrobial susceptibility trends for isolates from cattle with bovine respiratory disease in North America. *Journal of Clinical Microbiology*. 32: 725-731.

WETZSTEIN, H.G. 2005. Comparative mutant preventions of pradofloxacin and other veterinary fluoroquinolones indicate differing potentials in preventing selection of resistance. *Antimicrobial Agents and Chemotherapy*, 49: 4166-4173.

ZHAO, X. & DRLICA, K. 2001. Restricting the selection of the antibiotic–resistant mutants: a general strategy derived from flouroquinolone studies. *Clinical Infectious Disease* 33(3): S147-S156.

ZHAO, X & DRLICA, K. 2008. A unified anti-mutant dosing strategy. *Journal of Antimicrobial Chemotherapy*. 62: 434-436.

APPENDIX A. RAW DATA: SALMONELLA TYPHIMURIUM - ENROFLOXACIN

	SOURCE	Specie	Clinical	MIC (µg/ml)	MPC (µg/ml)
1	Abscess	Equine	x	0.25	0.5
2	Joint	Equine	x	0.25	4
3	Feaces	Equine	x	0.25	0.5
4	Joint	Equine	x	0.25	0.5
5	Blood culture	Equine	x	0.25	0.5
6	Feaces	Equine	x	0.25	0.5
7	Joint	Equine	x	0.25	0.5
8	Feaces	Equine	x	0.25	0.5
9	Feaces	Equine	x	0.25	0.5
10	Feaces	Equine	x	0.25	0.5
11	Joint	Equine	x	0.25	0.5
12	Feaces	Equine	x	0.25	0.5
13	Joint	Equine	x	0.25	0.5
14	Bone	Equine	x	0.25	0.5
15	Abscess	Equine	x	0.25	0.5
16	Feaces	Equine	x	0.25	0.5
17	Control				
18	Feaces	Equine	x	0.25	0.5
19	Feaces	Equine	x	0.25	4
20	Feaces	Equine	x	0.25	0.5
21	Joint	Equine	x	0.25	0.5
22	Feaces	Equine	x	0.25	0.5
23	Abscess	Equine	x	0.25	4
24	Joint	Equine	x	0.25	4
25	Feaces	Equine	x	0.25	4
26	Abscess	Equine	x	0.25	0.5
27	Feaces	Equine	x	0.25	0.5
28	Feaces	Equine	x	0.25	0.5

APPENDIX B. RAW DATA: PASTEURELLA MULTOCIDA - FLORFENICOL

SAMPLE	Specie	Source	Clinical	Survey	MIC (µg/ml)	MPC (µg/ml)
1	Bovine	Lung	x		0.25	8
2	Bovine	Trans	x		<0.25	<2
3	Bovine	Trans		x	0.5	<2
4	Bovine	Lung	x		<0.25	2
5	Bovine	Trans	x		<0.25	<2
6	Bovine	Trans	x		2	8
7	Bovine	Trans		x	1	8
8	Bovine	Trans		x	1	<2
9	Bovine	Trans		x	<0.25	<2
10	Bovine	Trans		x	1	<2
11	Bovine	Trans		x	2	<2
12	Bovine	Trans		x	2	<2
13	Bovine	Trans		x	1	<2
14	Bovine	Trans		x	<0.25	<2
15	Porcine	Lung	x		2	8
16	Bovine	Trans		x	0.5	32
17	Bovine	Lung	x		0.5	16
18	Porcine	Lung	x		0.25	<2
19	Bovine	Trans		x	0.5	<2
20	Bovine	Trans		x	0.5	<2
21	Bovine	Trans		x	0.5	<2
22	Bovine	Lung	x		<0.25	2
23	Porcine	Lung	x		1	<2
24	Bovine	Lung	x		0.25	32
25	Bovine	Lung		x	0.5	<2
26	Bovine	Lung	x		0.5	16
27	Control					
28	Bovine	Lung	x		0.25	16
29	Bovine	Lung	x		0.25	16
30	Bovine	Lung	x		2	32

APPENDIX C. RAW DATA: PASTEURELLA MULTOCIDA - OXYTETRACYCLINE

SAMPLE	Specie	Source	Clinical	Survey	MIC (µg/ml)	MPC (µg/ml)
1	Bovine	Lung	x		4	16
2	Bovine	Trans	x		<0.5	4
3	Bovine	Trans		x	>8	16
4	Bovine	Lung	x		1	<1
5	Bovine	Trans	x		>8	16
6	Bovine	Trans	x		>8	16
7	Bovine	Trans		x	>8	16
8	Bovine	Trans		x	8	16
9	Bovine	Trans		x	<0.5	1
10	Bovine	Trans		x	<0.5	4
11	Bovine	Trans		x	1	1
12	Bovine	Trans		x	2	1
13	Bovine	Trans		x	>8	16
14	Bovine	Trans		x	<0.5	4
15	Porcine	Lung	x		1	16
16	Bovine	Trans		x	<0.5	16
17	Bovine	Lung	x		8	16
18	Porcine	Lung	x		2	4
19	Bovine	Trans		x	8	2
20	Bovine	Trans		x	0.5	1
21	Bovine	Trans		x	8	8
22	Bovine	Lung	x		>8	16
23	Porcine	Lung	x		1	1
24	Bovine	Lung	x		2	16
25	Bovine	Lung		x	2	16
26	Bovine	Lung	x		8	16
27	Control					
28	Bovine	Lung	x		8	16
29	Bovine	Lung	x		1	16
30	Bovine	Lung	x		>0.5	4

APPENDIX D: OXYTETRACYCLINE CERTIFICATE OF ANALYSIS

SIGMA-ALDRICH®

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Certificate of Analysis

Product Name:	OXYTETRACYCLINE DIHYDRATE SigmaUltra
Product Number:	O4636
Product Brand:	Sigma
Molecular Formula:	$C_{22}H_{24}N_2O_9 \bullet 2H_2O$
Molecular Mass:	496.46
CAS Number:	6153-64-6

TEST	SPECIFICATION	LOT 1421707V RESULTS
APPEARANCE (COLOR)	LIGHT YELLOW TO YELLOW-TAN	SLIGHTLY YELLOW
APPEARANCE (FORM)	POWDER	POWDER
PURITY (TLC AREA %)	≥99 %	99.4 %
SOLUBILITY (COLOR)	YELLOW TO YELLOW-ORANGE	VERY DEEP YELLOW
SOLUBILITY (TURBIDITY)	SOLUBLE	CLEAR (<3.5 NTU)
SOLUBILITY (METHOD)	0.1M IN 1.0M HYDROCHLORIC ACID	0.1M IN 1.0M HYDROCHLORIC ACID
RESIDUE ON IGNITION	≤ 1.0 %	< 0.05 %
MISCELLANEOUS TESTS	INSOLUBLE MATTER ≤0.1 %	CORRESPONDS
METAL TRACE ANALYSIS (ICP)	CORRESPONDS TO REQUIREMENTS	PASSED
ALUMINIUM (ICP)	≤ 10 MG/KG	< 10 MG/KG
CALCIUM (ICP)	≤ 2000 MG/KG	< 2000 MG/KG
COPPER (ICP)	≤ 5 MG/KG	< 5 MG/KG
IRON (ICP)	≤ 20 MG/KG	< 20 MG/KG
POTASSIUM (ICP)	≤ 50 MG/KG	< 50 MG/KG
MAGNESIUM (ICP)	≤ 50 MG/KG	< 50 MG/KG
SODIUM (ICP)	≤ 100 MG/KG	< 100 MG/KG
LEAD (ICP)	≤ 10 MG/KG	< 10 MG/KG

SIGMA-ALDRICH®

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Certificate of Analysis

ZINC (ICP)	≤ 5 MG/KG	< 5 MG/KG
TOTAL PHOSPHORUS AS PO4 (ICP)	≤ 10 MG/KG	< 10 MG/KG
TOTAL SULFUR AS SO4 (ICP)	≤ 500 MG/KG	< 500 MG/KG
CHLORIDE (CL)	≤ 500 MG/KG	< 500 MG/KG
QC RELEASE DATE	15/JAN/09	

Edeltraud Schwärzler, Manager
Quality Control
Buchs, Switzerland

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APPENDIX E: FLORFENICOL CERTIFICATE OF ANALYSIS

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sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Product Specification

Product Name:
Florfenicol – analytical standard, for drug analysis

Product Number:	**F1427**
CAS Number:	73231-34-2
MDL:	MFCD00864834
Formula:	C12H14Cl2FNO4S
Formula Weight:	358.21 g/mol

TEST	**Specification**
Appearance (Color)	White to Off-White
Appearance (Form)	Powder
Solubility (Color)	Colorless to Light Yellow
Solubility (Turbidity)	Clear
50mg/mL, EtOH	
Proton NMR spectrum	Conforms to Structure
13C NMR SPECTRUM	Conforms to Structure
Carbon	39.4 - 41.0 %
Nitrogen	3.7 - 4.1 %
Purity (TLC)	≥ 98 %

Specification: PRD.0.ZQ5.10000012561

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APPENDIX F: ENROFLOXACIN CERTIFICATE OF ANALYSIS

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Tel: +41 81 755 2511 Fax: +41 81 756 5449

Certificate of Analysis

Product Name:	ENROFLOXACIN
	-
Product Number:	17849
Product Brand:	Fluka
Molecular Formula:	$C_{19}H_{22}FN_3O_3$
Molecular Mass:	359.39
CAS Number:	93106-60-6

TEST	SPECIFICATION	LOT BCBG4783V RESULTS
APPEARANCE (COLOR)	WHITE TO FAINTLY YELLOW	FAINTLY YELLOW
APPEARANCE (FORM)	POWDER	POWDER
PURITY (HPLC AREA %)	≥ 98.0 %	99.1 %
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

QC RELEASE DATE 15/SEP/11

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

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APPENDIX G: ATCC SALMONELLA

Certificate of Analysis: Lyophilized Microorganism Specification and Performance Upon Release

Specifications
Microorganism Name: Salmonella enterica subsp. enterica serovar Typhimurium
Catalog Number: 0421
Lot Number: 421-65
Reference Number: ATCC® 13311™*
Purity: < 0.1% Total Pellet CFU
Recovery: > 1000 CFUs per Pellet
Passage from Reference: 4

Expiration Date: 2012/08
Release Information:
Quality Control Technologist: Megan Mum
Release Date: 2010/10/20

Performance

Macrosopic Features:
Medium, gray/white, circular, convex colonies.
Microscopic Features:
Gram negative straight rod.

Medium:
SBAP
Method:
Gram Stain (1)

Vitek GN (1)

Phenotypic Features	Results
Ala-Phe-Pro-ARYLAMIDASE	-
ADONITOL	-
L-Pyrrolydonyl-ARYLAMIDASE	-
L-ARABITOL	-
D-CELLOBIOSE	-
BETA-GALACTOSIDASE	-
H2S PRODUCTION	+
BETA-N-ACETYL-GLUCOSAMINIDASE	-
Glutamyl Arylamidase pNA	-
D-GLUCOSE	+
GAMMA-GLUTAMYL-TRANSFERASE	-
FERMENTATION/GLUCOSE	-
BETA-GLUCOSIDASE	-
D-MALTOSE	+
D-MANNITOL	+
D-MANNOSE	+
BETA-XYLOSIDASE	-
BETA-Alanine arylamidase pNA	-
L-Proline ARYLAMIDASE	-
LIPASE	-
PALATINOSE	-
Tyrosine ARYLAMIDASE	+
UREASE	-
D-SORBITOL	+
SACCHAROSE/SUCROSE	-
D-TAGATOSE	+
D-TREHALOSE	+
CITRATE (SODIUM)	+
MALONATE	-
5-KETO-D-GLUCONATE	-
L-LACTATE alkalinization	+
ALPHA-GLUCOSIDASE	-
SUCCINATE alkalinization	+
BETA-N-ACETYL-GALACTOSAMINIDASE	-
ALPHA-GALACTOSIDASE	+
PHOSPHATASE	+
Glycine ARYLAMIDASE	-
ORNITHINE DECARBOXYLASE	+
LYSINE DECARBOXYLASE	+
L-HISTIDINE assimilation	-
COURMARATE	+
BETA-GLUCORONIDASE	-
O/129 RESISTANCE (comp.vibrio.)	-
Glu-Gly-Arg-ARYLAMIDASE	-
L-MALATE assimilation	-
ELLMAN	-
L-LACTATE assimilation	-

Other Features/ Challenges: Results

(1) Oxidase(Kovacs): negative
Hektoen Enteric agar: good growth, blue-green colonies with black centers
(1) Salmonella O antiserum Factor O:4 (included in group B): positive
(1) Salmonella O antiserum Factor O:5 (included in group B): positive
(1) Salmonella O antiserum Factor O:12 (included in group B): positive

Brad Goskowicz, President
AUTHORIZED SIGNATURE

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Certificate of Analysis: Lyophilized Microorganism Specification and Performance Upon Release

Specifications **Microorganism Name:** Salmonella enterica subsp. enterica serovar Typhimurium **Catalog Number:** 0421 **Lot Number:** 421-85 **Reference Number:** ATCC® 13311™* **Purity:** < 0.1% Total Pellet CFU **Recovery:** > 1000 CFUs per Pellet **Passage from Reference:** 4	**Expiration Date:** 2012/08 **Release Information:** **Quality Control Technologist:** Megan Murn **Release Date:** 2010/10/20

Disclaimer: The last digit(s) of the lot number appearing on the packing slip is merely a packaging event number. The lot number displayed on this certificate is the actual base lot number.

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TESTING CERT #2655.01

(1) These tests are accredited to ISO/IEC 17025:2005.

APPENDIX H: ATCC PASTEURELLA MULTOCIDA

Certificate of Analysis: Lyophilized Microorganism Specification and Performance Upon Release

Specifications
Microorganism Name: Pasteurella multocida subsp. multocida
Catalog Number: 0668
Lot Number: 668-43
Reference Number: ATCC® 12945™*
Purity: < 0.1% Total Pellet CFU
Recovery: > 1000 CFUs per Pellet
Passage from Reference: 4

Expiration Date: 2013/01
Release Information:
Quality Control Technologist: Megan Mum
Release Date: 2011/4/12

Performance

Macroscopic Features:
Medium to large, white to gray, circular to irregular, entire edge, glistening, mucoid; both opaque and translucent colonies.

Medium:
SBAP

Microscopic Features:
Gram negative coccobacilli or short rods, may be pleomorphic.

Method:
Gram Stain (1)

Vitek GN (1)

Phenotypic Features	Results
Ala-Phe-Pro-ARYLAMIDASE	-
ADONITOL	-
L-Pyrrolydonyl-ARYLAMIDASE	-
L-ARABITOL	-
D-CELLOBIOSE	-
BETA-GALACTOSIDASE	-
H2S PRODUCTION	-
BETA-N-ACETYL-GLUCOSAMINIDASE	-
Glutamyl Arylamidase pNA	-
D-GLUCOSE	+
GAMMA-GLUTAMYL-TRANSFERASE	-
FERMENTATION/GLUCOSE	-
BETA-GLUCOSIDASE	-
D-MALTOSE	-
D-MANNITOL	+
D-MANNOSE	+
BETA-XYLOSIDASE	-
BETA-Alanine arylamidase pNA	-
L-Proline ARYLAMIDASE	-
LIPASE	-
PALATINOSE	-
Tyrosine ARYLAMIDASE	+
UREASE	-
D-SORBITOL	-
SACCHAROSE/SUCROSE	+
D-TAGATOSE	-
D-TREHALOSE	-
CITRATE (SODIUM)	-
MALONATE	-
5-KETO-D-GLUCONATE	-
L-LACTATE alkalinization	-
ALPHA-GLUCOSIDASE	-
SUCCINATE alkalinization	-
BETA-N-ACETYL-GALACTOSAMINIDASE	-
ALPHA-GALACTOSIDASE	-
PHOSPHATASE	+
Glycine ARYLAMIDASE	-
ORNITHINE DECARBOXYLASE	-
LYSINE DECARBOXYLASE	-
L-HISTIDINE assimilation	-
COURMARATE	-
BETA-GLUCORONIDASE	-
O/129 RESISTANCE (comp.vibrio.)	-
Glu-Gly-Arg-ARYLAMIDASE	-
L-MALATE assimilation	-
ELLMAN	+
L-LACTATE assimilation	-

Other Features/ Challenges: Results
(1) Oxidase (Kovacs): weak positive

Brad Goskowicz, President
AUTHORIZED SIGNATURE

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Certificate of Analysis: Lyophilized Microorganism Specification and Performance Upon Release

Specifications	Expiration Date: 2013/01
Microorganism Name: Pasteurella multocida subsp. multocida	**Release Information:**
Catalog Number: 0668	**Quality Control Technologist:** Megan Murn
Lot Number: 668-43	**Release Date:** 2011/4/12
Reference Number: ATCC® 12945™*	
Purity: < 0.1% Total Pellet CFU	
Recovery: > 1000 CFUs per Pellet	
Passage from Reference: 4	

Disclaimer: The last digit(s) of the lot number appearing on the packing slip is merely a packaging event number. The lot number displayed on this certificate is the actual base lot number.

Note for Vitek©: Although the Vitek© panel uses many conventional tests, the unique environment of the card, combined with the short incubation period, may produce results that differ from published results obtained by other methods.

⚠ **Refer to the enclosed product insert for instructions, intended use and hazard/safety information.**

Individual products are traceable to a recognized culture collection.

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(1) These tests are accredited to ISO/IEC 17025:2005.

TESTING CERT #2655.01