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Pharmacokinetics of ceftiofur crystalline free acid in pigs vaccinated against, challenged with, or vaccinated against and challenged with PRRSv

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Pharmacokinetics of ceftiofur crystalline free acid in pigs vaccinated against, challenged with, or vaccinated against and challenged with PRRSv

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Preventive Medicine

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Iowa State University

Ames, Iowa

2015

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NOMENCLATURE

ANOVA	Analysis of variance
APP	*Actinobacillus pleuropneumoniae*
AUC	Area under the curve, for a drug concentration versus time plot
AUC % extrap	Area under the curve extrapolated for AUC_{inf}
$AUC_{0-\infty}$	Area under the curve, time zero to infinity
AUC_{0-last}	Area under the curve, time zero to last time point
B. bronchiseptica	*Bordetella bronchiseptica*
DCA	Desfuroylceftiofuracetamide
DTE	Dithioerythritol
C_{max}	Maximum plasma concentration
CCFA	Ceftiofur crystalline free acid
Cl/F	Plasma clearance per faction of dose absorbed
E. coli	*Escherichia coli*
F	Fraction of dose absorbed
IM	Intramuscular
IV	Intravenous
K_{el}	Elimination constant
nsp	Non-structural protein
ORF	Open reading frame
LPS	Lipopolysaccharide
P. multocida	*Pasteurella multocida*

PCR	Polymerase chain reaction
PK	Pharmacokinetic(s)
PRRS MLV	Porcine reproductive and respiratory syndrome modified live virus
PRRSv	Porcine reproductive and respiratory syndrome virus
QC	Quality control
RT-PCR	Reverse transcription polymerase chain reaction
S. suis	*Streptococcus suis*
T_{max}	Time to maximum concentration
Tukey's HSD	Tukey's honest significant difference test
Vz/F	Apparent volume of distribution per fraction of dose absorbed
Vx	Vaccine, in reference to vaccinated treatment group
λ_z	Terminal slope of drug concentration versus time curve

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Locke Karriker, and my committee members, Dr. Hans Coetzee, and Dr. Tim Day, for their guidance and support throughout the course of this research. Dr. Karriker has been an amazing mentor, taking my education further than I ever expected. I was absolutely privileged to work under him. The wisdom I gained will remain with me for life.

In addition, I would also like to thank swine-focused faculty of Iowa State University College of Veterinary Medicine. Their commitment to students and education paralleled with research show exceptional character. They are unequivocally representative of what every university professor should be.

Of final importance, I thank my family. My parents established me with a foundation of work ethic and faith. My wife, Ashley, offered hours of patience, respect, love, and support. My daughter, Bristol, gave me a unique piece of joy every day.

ABSTRACT

Antimicrobial treatment regimens are generally based on pharmacokinetic data established in healthy animals. Likewise, in food animals, antimicrobial withdrawal times are based on pharmacokinetic data from healthy animals. In clinical practice, antimicrobials are therapeutically used in disease challenged animals. There is limited literature addressing the question of pharmacokinetic changes in diseased animals. Considering swine, there are approximately 17 searchable peer reviewed studies addressing disease influence on antimicrobial pharmacokinetics. Across those studies there are about 28 disease-antimicrobial interactions evaluated, of which 21 find that disease changes the pharmacokinetics of the evaluated antimicrobial. None of the current studies address the influence vaccination may have on preserving antibiotic pharmacokinetics in the face of disease challenge.

Original research was performed to evaluate the pharmacokinetics of ceftiofur crystalline free acid in pigs vaccinated against, challenged with, or vaccinated against and challenged with PRRSv. The original research hypothesized that PRRSv wild-type challenge would change pharmacokinetics and previous vaccination would have no effect on pharmacokinetic variables. Previous research had shown ceftiofur pharmacokinetics change with PRRSv infection. The PRRSv vaccine investigated is a commonly used and commercially available modified live virus. The present work found that a wild-type PRRSv infection resulted in a lower AUC_{0-last}, higher Cl/F and higher Vz/F. The present work also determined that the modified live virus used for vaccination did not result in any pharmacokinetic changes, and vaccination with the modified live virus prevented pharmacokinetic changes in pigs that were subsequently challenged with a wild-type virus.

Disease or infection of a virulent organism does not always change antimicrobial pharmacokinetics. Pharmacokinetic changes seem dependent on specific antimicrobial and specific organism. Available research demonstrates PRRSv infection does change pharmacokinetics of ceftiofur drugs. Original research reported in this thesis suggests a modified live PRRSv vaccination has the potential to prevent ceftiofur pharmacokinetic changes that occur in the face of a wild-type PRRSv challenges. This information may be clinically applied by following labeled pharmacokinetic parameters of ceftiofur crystalline free acid in pigs vaccinated with a modified live PRRSv, regardless of wild-type PRRSv challenge.

CHAPTER 1

GENERAL INTRODUCTION

Thesis introduction

Antimicrobial dosing regimens are based on pharmacokinetic (PK) data. PK data is typically derived from healthy animals. Veterinarians often follow suggested dosing regimens when therapeutically treating diseased animals. In swine, there is a moderate amount of research observing certain antimicrobial PK changes in diseased animals. The PK changes observed in diseased animals have potential to change antimicrobial efficacy and ultimately clinical outcome. Considering food animals, alterations in pharmacokinetics can also have influence on withdrawal times.

There are two studies specifically investigating how PRRSv infection changes antimicrobial PK. Both studies evaluate the PK of ceftiofur hydrochloride. One study used solely a PRRSv challenge model (Tantituvanont et al., 2008), and the other study used a PRRSv and *Streptococcus suis* co-challenge model (Day et al., 2015). Both studies observed ceftiofur PK changes where disease challenged resulted in decreased C_{max}, decreased AUC, increased Cl/F, and increased Vz/F. This previous research instigated questions about the influence of PRRSv on longer acting ceftiofur preparations and the role PRRSv vaccination might have on disease altered PK parameters. The goal of this thesis was to understand the pharmacokinetics of ceftiofur crystalline free acid in pigs vaccinated against, challenged with, or vaccinated against and challenged with PRRSv.

Thesis organization

This thesis consists of five chapters. Chapter 1 is thesis introduction, organization, and objectives. Chapter 2 is a brief introduction to existing PK research that focuses on diseased swine. Chapter 2 focuses on proposed or defined mechanisms that support observed PK changes that exist in animals. Chapter 3 is titled "Vaccination mitigates the impact PRRSv infection has on the pharmacokinetics of ceftiofur crystalline free acid in pigs," and will be submitted to the *Journal of Veterinary Pharmacology and Therapeutics* for publication. Chapter 4 includes additional methods and results from the original research explained in Chapter 3. Chapter 5 is a summary and conclusion with brief review on possible PRRSv interactions that may contribute to observed PK changes.

Thesis objectives

The objectives of Chapter 2 were, 1) to offer an updated summary to a previously completed literature review focusing on antimicrobial PK changes in swine, and 2) Review current literature to summarize possible mechanisms responsible for disease changing antimicrobial pharmacokinetics. The objectives of Chapter 3 were defined as part of the original research study as being 1) determine if PRRS MLV alone impacts the pharmacokinetic profile of ceftiofur crystalline free acid in pigs, 2) determine if PRRS MLV vaccination prevents PK impacts when vaccinated pigs are challenged with a wild-type PRRSv. The objective of Chapter 4 was to include and explain additional methods and results of Chapter 3; though the information was important to the original research, it was truncated from Chapter 3 for publication purposes. The objective of Chapter 5 was to identify possible mechanisms specifically related to PRRSv that may contribute to observed antimicrobial PK changes. Chapter 5 also serves as a final conclusion for the thesis.

CHAPTER 2

ANTIMICROBIAL PHARMACOKINETICS IN DISEASED SWINE AND PROPOSED

MECHANISMS FOR OBSERVED CHANGES

Introduction

Considering swine, there are approximately 17 peer reviewed searchable studies

addressing disease influence on antimicrobial pharmacokinetics. Across those studies there are

about 28 disease-antimicrobial interactions evaluated. Among the 28 interactions, 21 find that

disease changes the pharmacokinetics of the evaluated antimicrobial. Table 1 is adapted,

updated, and modified from Day (2014), and it summarizes the studies among swine that

evaluate disease influence on antimicrobial pharmacokinetics. Each study shares a common

objective of determining the pharmacokinetic influence disease might have on a given

antimicrobial administered to swine. Ultimately, it is difficult to recognize a common theme for

disease influence on antimicrobial pharmacokinetics. Yet, each study has a unique disease-

antimicrobial interaction.

The available research spans 36 years (Ladefoged, 1979 to Day et al., 2015) and several

research groups. When evaluating these studies it is important to be cognizant of differences. The

following is a brief list of points of variability that exists in study design: breed and sex of pigs,

methods of disease introduction, virulence of organism used, use of actual organism or products

produced by organism, timing of disease inoculation prior to pharmacokinetic assessment,

verification of disease state, blood collection, number and duration of blood collections for PK

analysis, methods of drug extraction and quantification, pharmacokinetic modeling and

programs, pharmacokinetic parameters reported, statistical analysis and programs. Beyond

characteristics of explicit study design, there are points of variation that are not mentioned,

which could include the following: conditions of the animals housing, daily care of the animals,

methods for handling and restraining the animals, and laboratory protocols for handling samples.

The listed areas of variation are not complete but rather suggest points where differences can

change interpretable results. These areas of variation make it difficult for general applications

and interpretations. However, if studies are individually evaluated in context of all available

information, clinical interpretations and applications may still be made.

Within each journal article evaluating disease influence on antimicrobial

pharmacokinetics, authors offer explanations as to why pharmacokinetic changes are or are not

observed. Some explanations are derived from original research conducted within the given

study. Some explanations reference previously published articles. Some explanations are

speculation based on known physiological mechanisms. The remainder of this thesis chapter will

review some of the explanations and theories other authors have offered for the mechanisms of

altered pharmacokinetics in a disease state.

Renal function

Kidneys serve as a major excretory route from many drugs. Renal drug excretion may

occur through glomerular filtration, tubular secretion, or passive diffusion (Riviere, 2009a).

Renal reabsorption may also occur, transporting drugs back into the vasculature. Plasma

clearance is the most probable PK parameter to show changes with altered renal function.

Mengelers et al. (1995) measured urine volume, pH, and drug content in pigs challenged

with APP toxin and administered sulfadimethoxine/trimethoprim or

sulfamethoxazole/trimethoprim. Disease challenge increased plasma drug clearance of

trimethoprim in both combinations, but there were no clearance changes of sulfadimethoxine or sulfonamide. In both drug combinations, amount of trimethoprim excreted in urine was higher in APP toxin challenged pigs. Amounts of sulfadimethoxine, sulfamethoxazole, and metabolites excreted in urine did not differ between challenge and control pigs. Urine volume and pH showed no difference between any treatment groups. The authors could not offer an explanation for the observed increase of trimethoprim clearance in APP toxin challenged pigs. However, they did conclude that urine pH and volume did not contribute to the observed clearance changes.

Post et al. measured urine content of enrofloxacin and ciprofloxacin when studying enrofloxacin PK in pigs challenged with APP (Post el al., 2002) or *E. coli* LPS (Post et al., 2003). APP challenge resulted in no change of plasma drug clearance. *E. coli* LPS challenge resulted in decreased plasma drug clearance. Dexamethasone, chosen for anti-inflammatory properties, was included in both studies as an effect of a 2x2 factorial design. In both studies, enrofloxacin/creatinine ratio and ciprofloxacin/creatinine ratio were used to estimate drug clearance by renal excretion. Creatinine normalization was utilized to avoid confounding that may occur from increased drinking and urination resultant of dexamethasone usage. APP infection did result in a lower enrofloxacin/creatinine and ciprofloxacin/creatinine ratios at 24 hours but not at 48hrs. Reference is made indicating APP does not change hepatic metabolism or renal blood flow, as evidenced by indole cyanine green and creatinine clearance (Monshouwer et al., 1995). Post et al. concluded APP may inhibit renal tubular secretion or enhance reabsorption. However, the authors acknowledge that this observation may only be transient. Considering *E. coli* LPS challenged pigs, total enrofloxacin equivalents in urine was greater 48 to 72 hours after drug administration. This increase was not observed in the *E. coli* LPS challenged pigs also receiving dexamethasone, which led authors to suggest that the increased renal excretion of drug

was likely a result of inflammation. However, the total urine equivalents for the entire study did not differ from control animals. These observations were explained to be a result of decreased plasma clearance in LPS challenged pigs.

Considering pharmacokinetics in disease challenged swine, other studies addressing renal function do not have original research but have references and suggestions. Zeng and Fung (1990) observed increased clearance of penicillin G in *Streptococcus suis* challenged pigs. They suggested increased clearance could be a result of fever and consequent increased renal blood flow, which was shown in previous literature (van Miert, 1985).

Jensen et al. (2006) studied oral amoxicillin pharmacokinetics in *E.coli* challenged pigs, and found, relative to control pigs, disease decreased then increased clearance on day one and day two after amoxicillin administration, respectively. They suggested *E. coli* diarrhea could have changed hydration status possibly resulting in renal dysfunction changes between day one and two. Dehydration was not clinically noted. They also referenced a study demonstrating *E. coli* endotoxin being able to decrease renal clearance in dogs (Marier et al., 2001).

Yuan et al. (1997) completed a study showing an increased ampicillin clearance but a decreased sulfadimidine clearance in *Streptococcus suum* challenged pigs. With knowledge of ampicillin being eliminated by active tubular secretion, they suggest *Streptococcus suum* infection increases renal tubular secretion, resulting in greater ampicillin clearance. They attributed decreased sulfadimidine clearance to decreased glomerular filtration, which is presumed to be decreased in *Streptococcus suum* infection.

Considering the importance of renal function, there is little research addressing renal changes and influences on pharmacokinetics in diseased swine. From available research (Mengelers et al. 1995; Post et al. 2002; Post et al. 2003), kidneys of disease challenged swine

appear to maintain functional capacity with none to minor changes in antimicrobial pharmacokinetics. However this concept has high potential to be disease and drug specific.

Plasma Protein binding

Clinical pharmacokinetics, especially those used among swine, are based upon drug concentrations in the vasculature. With studies pertaining to swine there are three general routes a drug will enter the vasculature 1) direct intravenous injection, 2) absorption from an intramuscular or subcutaneous injection site, or 3) absorption from the gastrointestinal tract. Once in the vasculature, many classes of drugs are subject to some degree of protein binding. Albumin is often considered a highly important protein for drug protein binding to facilitate distribution. Covalent, noncovalent, and ionic binding are examples of common interactions a drug might have with a plasma protein. If a drug is bound to a plasma protein it is generally not considered available for distribution or excretion. It is the non-bound drug that is available for distribution into tissues or excretory elimination from the body (Riviere, 2009a). Change in plasma proteins is one theory for observed pharmacokinetic changes in disease pigs.

Mengelers et al. (1995) measured protein binding by ultrafiltration, when evaluating sulfadimethoxine/trimethoprim or sulfamethoxazole/trimethoprim in APP toxin challenged pigs. For all treatment groups, proportion of protein binding in the vasculature for sulfadimethoxine, sulfamethoxazole, and trimethoprim was 94-99%, 45-56%, and 40-50%, respectively. Protein binding of all drugs and metabolites was consistently lower in the disease challenged pigs, but the difference was not statistically significant.

Lindercrona et al. (2000) studied danofloxacin pharmacokinetics in pigs challenged with *Salmonella typhimurium.* They found that challenged pigs had a significantly higher percent of

protein binding, 53±8% versus 44±8% in control animals. The authors offered no explanation for the higher protein binding in disease challenged animals.

Agerso et al. (1998) studied amoxicillin pharmacokinetics in APP challenged pigs. Amoxicillin protein binding in plasma and bronchial secretions was measured by ultrafiltration. The results were not compared to control animals. They found amoxicillin protein binding to be 17±8% in plasma and 13±6% in bronchial secretions. They also concluded that similar concentrations of amoxicillin in pleural fluid and plasma indicate the protein binding in pleura fluid is similar to the protein binding of plasma.

Tantituvanont et al. (2009) observed increased volume of distribution and clearance in PRRSv infected pigs. They did not directly measure protein binding or plasma proteins. However, they did reference the possible decline in plasma albumin with pneumonia (Parra et al., 2006), which would leave more non-protein bound drug available for distribution or excretion. Others have simply alluded to the concept of protein binding. Zeng and Fung (1990) suggest lower plasma protein binding could be the cause of their observed increased volume of distribution for penicillin G in *S. suis* challenged pigs. Yuan et al. (1997) suggests decreased plasma proteins may be an explanation to their observed increase in volume of distribution and clearance of Ampicillin in *Strep suum* infected pigs.

Vascular permeability, flow, and pH

As previously mentioned, the vasculature is commonly considered the central compartment for pharmacokinetics. Reasonably, changes of the vasculature would be expected to change absorption, distribution, metabolism, or excretion for a given drug. There are several plausible physiological explanations of how disease may change vasculature to influence

pharmacokinetics. However, there is little original research measuring these changes directly in swine.

Agerso et al. (1998) studied pharmacokinetics and tissue distribution of IV amoxicillin in APP challenged pigs. There was an increased volume of distribution of infected animals compared to the controls. They concluded this increased volume of distribution was a result of change in peripheral compartments and suggested a possible cause being dilated capillaries with increased vascular supply to the infected region. The authors also reference the idea that infection induced fever and subsequent shivering could divert blood flow and alter pharmacokinetics (Blatteis et al., 1988). Agerso et al. (2000) also studied pharmacokinetics and tissue distribution of IM amoxicillin in *Salmonella typhimurium* challenged pigs. In this case amoxicillin tissue distribution of infected animals was numerically, not statistically, decreased. There was a statistically significant increase of mean residence time and volume of distribution in infected animals. They suggested dilated capillaries and altered blood flow as possible, but not ultimate, causes.

The concept of changes in blood pH has been another suggestion of how disease may change antimicrobial pharmacokinetics. Drug ionization is usually dependent on pH, and ionization influences the ability for a compound to cross biological membranes (Riviere, 2009a). Mengelers et al. (1995) measured urine pH when studying sulfadimethoxine/trimethoprim or sulfamethoxazole/trimethoprim in APP toxin challenged pigs. Urine pH has some potential to offer inference of blood pH. They did not find any difference between control and challenged pigs. Others have suggested the concept of pharmacokinetic changes being resultant of disease influenced vasculature pH, but there is no original research or referencing (Zeng and Fung, 1990; Agerso et al., 1998; Day, 2014).

Gastrointestinal absorption

Orally administered drugs are open to a unique area of influence. Disease impact on gastrointestinal function has changes mostly manifested in drug absorption. When considering the influence disease has on orally administered antimicrobial pharmacokinetics, it is important to recognize areas of variability. In experimental conditions the drug may be provided *ad libitum* in feed, *ad libitum* in water, placed in the oral cavity, or gavaged. In some studies, animals are fasted prior to drug administration.

Pijpers et al. (1991) evaluated oxytetracycline in fasted pigs challenged with APP toxin. They found disease resulted in increased AUC and Vd, while decreasing C_{max} and Cl. A suggested cause was delayed gastric emptying allowing more time for drug absorption. They referenced work in rats that demonstrated disease may delay gastric emptying and changed gastric pH (Leenen and van Miert, 1969; van Miert AS, De la Parra DA, 1970). However, they also acknowledged other aspects of disease can contribute to changes in drug absorption, referencing fever in calves changing absorption for an intramuscularly administered drug (Groothuis et al., 1980).

Jensen et al. studied *E. coli* challenged pigs administered amoxicillin by oral gavage (2004) and ad libitum in drinking water (2006). *E. coli* challenged pigs administered amoxicillin by oral gavage had a lower AUC and C_{max}, relative to control pigs. Studies in rats and people have demonstrated amoxicillin absorption occurs through a sodium exchange mechanism and passive diffusion (Westphal et al., 1995; Sugawara et al., 1990). *E.coli* diarrhea is due to a secretory sodium-chloride mechanism (Argenzio, 1984) and, to a lesser extent, loss of intestinal surface area (Vijtiuk et al., 1995). Using the aforementioned references, Jenson et al. (2004) suggested their observed pharmacokinetic changes are a result of amoxicillin absorption and *E.*

coli secretion competing for the same mechanism along with a general reduction of intestinal absorptive surface area. In the later study by Jensen et al. (2006), relative to control pigs, *E. coli* challenged pigs with *ad libitum* access to amoxicillin treated water had decreased AUC, decreased C_{max}, and deceased K_{el} one day after amoxicillin access, but conversely two days after amoxicillin access, AUC, C_{max}, and K_{el} were significantly increased relative to control. They again suggested a competitive mechanism and reduced intestinal surface area for day one PK changes. The shift seen from day one to two was assumed to be a result of partially saturating the absorptive capacity for amoxicillin by use of a large dose.

Godoy et al. (2011) studied pharmacokinetics of amoxicillin administered by *ad libitum* feed in pigs naturally infected with PRRSv. The study observed higher AUC and C_{max} in disease challenged pigs, when compared with non-diseased pigs. They suggested that a reduction in intestinal transit rate increased the amount of time the amoxicillin medicated feed was residing in the intestinal tract, allowing for more absorption. A rat study was sited to reference ampicillin bioavailability doubled with increasing time the drug set in the small intestine (Haruta et al., 1998). The authors also briefly mentioned blood flow, hepatic function, renal function, and hydration are potential causes for pharmacokinetic changes in diseased animals.

In addition to the physiologic drug-body interaction, animal behavior in a disease state may be a key point of influence. Sick animals may be lethargic and not be as ambitious to drink or eat. Pijpers et al. (1991) measured water and feed consumption and observed significant decreases in diseased pigs. Godoy et al. (2011) noted animal behavior is highly variable on the individual level and animal behavior in a disease state can decrease consumption of medicated *ad libitum* feed or water.

Medicated feed and water are common routes of antimicrobial therapy in commercial swine (Food and Drug Administration, 2013). As seen with other routes of medication, disease has potential to change orally administered pharmacokinetics in diseased swine. Functionality of the gastrointestinal tract is an added consideration when giving oral antimicrobials. Intestinal motility, altered pH, and possible interference with antimicrobial transmembrane transport are major areas discussed by the authors that studied oral antimicrobial pharmacokinetics in diseased pigs. Though not directly considered a pharmacokinetic parameter, disease induced lethargic behavior has the potential to be a significant factor in the ability of an *ad libitum* oral antimicrobial to make it to the target site in a diseased pig.

Other possible mechanisms changing pharmacokinetics in diseased swine

Considering swine literature, the preceding paragraphs include more prevalent ideas regarding mechanisms for antimicrobial pharmacokinetic changes seen in diseased challenged swine. However, there are a few other mechanistic suggestions. Considering inflammatory mediators, Post et al. (2002) measured IL-6 in APP challenge pigs given enrofloxacin. They found IL-6 elevated in challenged pigs, but there was no conclusion made from the observation. Lindercrona et al. (2000) suggested alteration in hepatic metabolism as a possible change for danofloxacin pharmacokinetics in *Salmonella typhimurium* challenges pigs. Lui et al., (2003) noticed no difference in florfenicol pharmacokinetics when given IV, IM, or orally to APP challenged pigs. They suggested the reason for no observed change stems from APP causing respiratory disease and not having much effect on liver or kidneys.

Summary on mechanisms changing pharmacokinetics in disease swine

There are numerous ways to dissect and categorize the possible mechanisms related to antimicrobial pharmacokinetic changes seen in diseased pigs. The best deduction is that disease

or infection has potential to change antimicrobial pharmacokinetics in a manner specific to the particular drug and disease combination. Given the present research in swine, a consistent generalized conclusion cannot be made. However, studies evaluating disease influence on antimicrobial pharmacokinetics in swine can be descriptively represented (Table 1). It is important to realize specific drug of interest and specific disease of interest is relevant. Within the pharmacokinetic studies, there is little original research attempting to explain mechanisms behind observed pharmacokinetic changes. In clinical settings, it is best to take original research, correlated with the conditions, and make the best decision for the specific circumstances.

CHAPTER 3

VACCINATION MITIGATES THE IMPACT PRRSV INFECTION HAS ON THE
PHARMACOKINETICS OF CEFTIOFUR CRYSTALLINE FREE ACID IN PIGS

To be submitted to *Journal of Veterinary Pharmacology and Therapeutics* for publication.

J.W. Sparks, L.A. Karriker, D.N. Day, L.W. Wulf, J.Q. Zhang, D.M. Madson, M.L. Stock, J.L. Bates, R. Gehring, J.F. Coetzee

Abstract

The pharmacokinetics of intramuscularly administered ceftiofur crystalline free acid (CCFA) were determined in pigs that were clinically healthy, vaccinated with a PRRS MLV, challenged with wild-type PRRSv VR-2385, or vaccinated with PRRS MLV and later challenged with wild-type PRRSv VR-2385. Animals were given a single dose intramuscularly at 5mg/kg bodyweight. Blood was collected at 0 (pre-treatment), 0.25, 0.5, 1, 6, 12, 24, 48, 96, 144, 192, and 240 hours post injection. Plasma was analyzed using liquid chromatography-mass spectrometry. Plasma concentration-time curves for each group were evaluated with noncompartmental modeling. Vaccination and challenge models were confirmed with strain specific RT-PCRs performed on lung or tonsil tissue. When compared to control animals, those receiving the PRRSv wild-type challenge had a lower $AUC_{0\text{-last}}$, higher Cl/F, and higher Vz/F. Control animals had no statistically significant differences from animals vaccinated with PRRS MLV alone or animals vaccinated with PRRS MLV and later challenged with wild-type PRRSv. Vaccination with PRRS MLV does not change the pharmacokinetics of CCFA, and our results suggest that when faced with wild-type PRRSv challenge, vaccination with PRRS MLV preserves pharmacokinetics of CCFA.

Introduction

Treatment regimens are generally based on pharmacokinetic (PK) profiles established in healthy animals. In clinical practice, antibiotics are therapeutically used in disease challenged animals. There is limited literature addressing the question of PK profile changes in diseased animals. Tantituvanont et al. (2008) studied pharmacokinetics of intramuscular injections of ceftiofur hydrochloride and found pigs intranasally challenged with porcine reproductive and respiratory syndrome virus (PRRSv) had decreased area under the curve (AUC), maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), and terminal elimination half-life ($T_{1/2z}$), while there was an increased clearance per fraction dose absorbed (Cl/F) and volume of distribution per fraction dose absorbed (Vz/F).

PRRSv has been estimated to annually cost the U.S. swine industry \$664 million (Holtkamp et al., 2013). PRRSv is known for interstitial pneumonia and reproductive failure (Rossow, 1998). PRRSv is also capable of causing increased susceptibility to secondary bacterial infections (Brockmeier et al., 2001; Thacker et al., 1999; Thanawongnuwech, et al., 2000).

Ingelvac[®] porcine reproductive and respiratory syndrome modified live virus (PRRS MLV) is an attenuated PRRSv strain. PRRS MLV is marketed and labeled to aid in disease reduction for both reproductive and respiratory forms of PRRSv. Vaccination with a PRRS MLV has been noted to reduce viremia, fever, and lung lesions when challenged with a virulent PRRSv (Johnson et al., 2004 and Nodelijk et al., 2001).

Ceftiofur crystalline free acid (CCFA) is a third generation cephalosporin.With swine, a majority of CCFA is metabolized to desfuroylceftiofur. CCFA, desfuroylceftiofur, and any metabolites possesing the β-lactam ring may be active against bacterial cell wall synthesis

(Beconi-Barker, 1995). CCFA for treatment of swine respiratory disease has proven efficacy against gram-postive and gram-negative bacteria, including β-lactamase producing strains (Food and Drug Administration, 2010). According to current labels, CCFA is a longer acting ceftiofur preparation when compared to ceftiofur hydrochloride, and ceftiofur sodium, with half-lives of 49.6, and 16.2, and 14.0 hours, respectively.

The objectives of this study were 1) determine if PRRS MLV alone impacts the pharmacokinetic profile of ceftiofur crystalline free acid in pigs, 2) determine if PRRS MLV vaccination prevents PK impacts when vaccinated pigs are challenged with a wild-type PRRSv.

Materials and methods

Animals and housing

Thirty-eight clinically healthy and PRRSv naïve crossbred barrows were sourced from a commercial herd. They were 3 to 4 weeks of age and weighed an averaged 9.8 kg at time of allotment. Each treatment group was housed in a separate biosecure and climate controlled room at Iowa State University Livestock Infectious Disease Isolation Facility. All rooms contained a single pen measuring $13.4m^2$ on a solid concrete floor. Pigs were allowed *ad libitum* access to a corn-soybean meal based diet without added antimicrobials. Pigs were allowed *ad libitum* access to water without antimicrobials by two nipple drinkers. All live animal care and procedures were in accordance with a protocol approved by Iowa State University's Institutional Animal Care and Use Committee (4-13-7542-S).

Experimental Design

An individual pig was considered the experimental unit. Pigs were blocked by weight and randomly allotted to one of four treatment groups. The first group served as control, receiving neither PRRS MLV nor PRRSv challenge (Control group, n=8). The second group received only

PRRS MLV vaccination five days prior to PK assessment (Vx group, n=10). The third group received only PRRSv wild-type challenge five days prior to PK assessment (Challenge group, n=10). The fourth group received PRRS MLV vaccination thirty-three days prior to PK assessment and received PRRSv wild-type challenge five days prior to PK assessment (Vx+Challenge group, n=10). The PK assessment for each group had a ten day duration. All pigs were necropsied at conclusion of the PK assessment. Necropsy was to harvest lung and tonsil tissues for diagnostics and viral characterization. A summary of study design is outlined in Table 2.

Veterinary products

CCFA (EXCEDE® FOR SWINE, Lot number 3F0184, Zoetis, Kalamazoo, MI, USA) was used as packaged, with each milliliter containing 100mg of ceftiofur equivalents in a Miglyol® and cottonseed oil based suspension. Injections were intramuscular in the post-auricular region of the right neck. On study day 0, administration of CCFA initiated the start of an individual pig's PK assessment.

Ingelvac® PRRS MLV (Serial number 245-D45, Boehringer Ingelheim Vetmedica, INC., St Joseph, MO, USA) was used as a modified live PRRSv vaccine. The vaccine was handled and reconstituted in accordance with labeled instructions. A 2mL PRRS MLV dose was administered using a 3mL syringe and 20gauge by1 inch needle (Monojet™ Syringe with Luer-Lock Tip and Veterinary Needle, Covidien, Mansfield, MA, USA). Injections were intramuscular in the post-auricular region of the left neck.

PRRSv challenge inoculum

The PRRSv challenge inoculum was prepared as previously reported (Day, 2014). Briefly, PRRSv isolate VR-2385 was initially recovered from a sow herd in southwestern Iowa

that experienced severe respiratory diseases in 3-16 weeks old pigs and late-term abortions in 1991 (Halbur et al., 1995). VR-2385 strain has been used for experimental infection or challenge in numerous studies (Doeschl-Wilson et al., 2009; Opriessnig et al., 2007; Thanawongnuwech et al., 1998). VR-2385 strain was propagated in Marc-145 cells, a clone of the African monkey kidney cell line MA-104 (Kim et al., 1993). The cells were cultured and maintained in RPMI-1640 Medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 0.05 mg/ml Gentamicin, 10 unit/ml penicillin, 10 µg/ml Streptomycin, and 0.25 µg/ml Amphotericin. Virus titration was performed in 96-well plates of Marc-145 cells by inoculating 10-fold serial dilutions of the virus (100 µl per well), triplicate per dilution. After 5 days inoculation, virus-specific cytopathic effect was recorded and the plates were subjected to immunofluorescence staining with PRRSv specific monoclonal antibodies conjugated to fluorescein isothiocyanate. The virus titers were determined according to the Reed and Muench method (Reed and Muench, 1938) and expressed as 50% tissue culture infective dose per milliliter (TCID50/ml). Five days prior to PK assessment, each pig in Challenge and Vx+Challenge groups received a 2mL intranasal dose at a concentration of $10^{5.6}$ $TCID_{50}$/mL using a 3mL syringe (Monojet™ Syringe with Luer-Slip Tip, Covidien, Mansfield, MA, USA).

Routine diagnostics and viral characterization

Prior to enrollment in the study, serum was collected from all pigs for PRRSv standard PCR and PRRSv antibody ELISA to confirm naïve status. Serum was collected from the Vx+Challenge group immediately prior to challenge, five days prior to PK assessment, for PRRSv antibody ELISA to confirm successful vaccine response. Serum was collected from all pigs on day 0, immediately prior to starting the PK assessment, for PRRSv standard PCR to confirm infection and/or vaccination status. Serum was collected from all pigs on day 9, prior to

necropsy, for PRRSv standard PCR to confirm infection and/or vaccination status at the end of the study. All serum was submitted to the Iowa State University Diagnostic Laboratory for routine testing. All samples for ELISA were tested with HerdChek X3® PRRSv antibody ELISA (IDEXX Laboratories, Inc., Westbrook, Maine) with a sample to positive ratio of ≥ 0.4 considered positive. Samples for PCR collected prior to allotment and on day 0 were tested using the Tetra-core PRRSv PCR with a cycle threshold of ≥ 40 considered positive. Samples for PCR collected prior to necropsy, on day 9, were tested using the Applied Biosystems PRRSv PCR with a cycle threshold of ≥ 37 considered positive. Both PCRs were the routine PRRSv PCRs offered to clients of the ISU VDL at the respective sample submission time points.

At necropsy, lung and tonsil tissues were collected for characterization of PRRSv viral strain. If lung samples were negative for PRRSv, tonsil was tested. Standard PRRSv PCR is unable to differentiate between the PRRS MLV and PRRSv VR-2385 viral strains. A reverse transcription PCR (RT-PCR) was developed to distinguish the Ingelvac® PRRS MLV from the PRRSv VR-2385 wild-type virus. The RT-PCR used virus-specific primers targeting both the ORF5 and the nsp2 genomic segments. The Ingelvac® PRRS MLV ORF5 was amplified and sequenced using the primers IngelvacMLV(ORF5)_77F and IngelvacMLV(ORF5)_574R with the thermal cycler conditions of 50°C 30 min, 95°C 15 min, 40 cycles (94°C 30 sec, 53°C 30 sec, 72°C 1 min), and 72°C 10 min. The PRRSv VR-2385 ORF5 was amplified and sequenced using the primers VR2385(ORF5)_77F and VR2385(ORF5)_574R with the thermal cycler conditions of 50°C 30 min, 95°C 15 min, 40 cycles (94°C 30 sec, 56°C 30 sec, 72°C 1 min), and 72°C 10 min. The Ingelvac® PRRS MLV nsp2 was amplified and sequenced using the primers IngelvacMLV_3007F and IngelvacMLV_3944R with the thermal cycler conditions of 50°C 30 min, 95°C 15 min, 40 cycles (94°C 30 sec, 60°C 30 sec, 72°C 1.5 min), and 72°C 10 min. The

PRRSv VR-2385 nsp2 was amplified and sequenced using the primers VR2385_3008F and

VR2385_3510R with the thermal cycler conditions of 50°C 30 min, 95°C 15 min, 40 cycles

(94°C 30 sec, 56°C 30 sec, 72°C 1 min), and 72°C 10 min.

<u>Pharmacokinetic drug administration and blood sampling</u>

Individual pigs were re-weighed within 12 hours of commencement of the PK

assessment. Weights were used to calculate the dose of 5.0mg ceftiofur equivalents per kilogram

body weight. Doses were rounded to the closest 1/10th of a milliliter. Injections were

intramuscular in the post-auricular region of the right neck using a 3mL syringe and 20 gauge by

1 inch needle (Monojet™ Syringe with Luer-Lock Tip and Veterinary Needle, Covidien,

Mansfield, Massachusetts, USA).

Blood was sampled 0 (pre-treatment), 0.25, 0.5, 1, 6, 12, 24, 48, 96,144,192, and 240

hours post-injection. Blood was collected from the jugular furrow using a 12mL syringe and 18

gauge by 1.5 inch needle (Monojet™ Syringe with Luer-Lock Tip and Veterinary Needle,

Covidien, Mansfield, MA, USA) while pigs were manually restrained. Blood was immediately

transferred to a 6.0 mL lithium heparin Vacutainer® (Becton, Dickinson and Company, Franklin

Lakes, New Jersey, USA), inverted at least five times, and placed in a cooler with ice. Samples

would remain in a cooler no longer than two hours before being centrifuged at 1000g and 4°C for

10 minutes. Using disposable transfer pipettes, plasma was transferred to individual cryovials.

Plasma was stored at -70°C until plasma analysis was performed.

<u>Plasma analysis</u>

Plasma concentrations of ceftiofur equivalents were determined using liquid

chromatography coupled with mass spectrometry (LC-MS). All laboratory personnel conducting

plasma analysis were blinded to treatment group. The LC-MS system consisted of an Accela

Pump and Autosampler, (Thermo Scientific, San Jose, CA, USA) coupled to an ion trap mass

spectrometer (LTQ XL, Thermo Scientific, San Jose, CA, USA). Ceftiofur residues (total

ceftiofur, ceftiofur equivalents) were determined by cleavage of ceftiofur, its metabolites, and

protein bound residues to desfuroylceftiofur with dithioerythritol (DTE) followed by

derivatization with iodoacetamide. The stable derivative, desfuroylceftiofuracetamide (DCA),

was then analyzed by LC-MS. Deuterated ceftiofur, d3-ceftiofur was used as the internal

standard which became d3-desfuroylceftiofuracetamide upon cleavage and derivatization.

Cleanup of the derivatized samples was performed by solid phase extraction (SPE) using Oasis

HLB cartridges (Waters Associates, Milford, MA, USA). Plasma samples, plasma calibrators,

and quality control (QC) samples, 200 µL, were treated with 3 mL of 0.5% DTE in borate buffer,

0.05 N, pH 9.0 after addition of 10 µL of a 10 ng/µL solution of the internal standard, d3-

ceftiofur. The samples were vortexed for 5 seconds and placed in a 50°C water bath for 15

minutes. Upon removal from the water bath and cooling to room temperature, 0.5 mL of 14%

iodoacetamide in phosphate buffer (0.025 M, pH 7) was added followed by the samples being

left in the dark for 30 minutes. Following derivatization, the samples were cleaned up on an

Oasis HLB SPE column (60 mg/3 mL) that was preconditioned with 1 mL of methanol followed

by 1 mL of water. The sample was then transferred to the SPE column and allowed to pass

slowly through the HLB column. The column was washed with a 1 mL portion of 5% (v/v)

solution of methanol in water. The column was then dried for 5 minutes with a flow of nitrogen.

Elution of the derivatized samples was then performed with two 0.75 mL portions of 5% (v/v)

acetic acid in acetonitrile. The eluate was dried at 50°C with a stream of nitrogen in a Turbovap

evaporator. The dry residue was reconstituted with 100 μL of 25% (v/v) acetonitrile in water and

vortexed, followed by 50 μL of water and vortexed. The tube contents were transferred to an

autosampler vial fitted with a glass insert. The injection volume was set to 20 μL. The mobile

phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile at a

flow rate of 0.225 mL/min. The mobile phase began at 10% B with a linear gradient to 95% B in

6 minutes, which was maintained for 1.5 minutes, followed by re-equilibration to 10% B.

Separation was achieved with a Titan C18 column, 100 mm x 2.1 mm, 1.9 μm particles (Sigma

Chemical Co., St. Louis, MO, USA) maintained at 45°C. DCA eluted at 3.3 minutes and the

internal standard, d3-desfuroylceftiofuracetamide at 3.3 minutes. Full scan MS of the

pseudomolecular ions of DCA (m/z 487) and the internal standard; d3-

desfuroylceftiofuracetamide (m/z 490) was used for analyte detection. The sum of the intensities

of ions at m/z of 197, 241, 324, and 396 were used for DCA quantitation. The internal standard

was quantitated with the sum of the ion intensities at m/z of 200, 244, 327, and 399. Sequences

consisting of plasma blanks, calibration spikes, QC samples, and porcine plasma samples were

batch processed with a processing method developed in the Xcalibur software (Thermo

Scientific, San Jose, CA, USA). The processing method automatically identified and integrated

each peak in each sample and calculated the calibration curve based on a weighted (1/X)

quadratic fit. Plasma concentrations of ceftiofur in unknown samples were calculated by the

Xcalibur software based on the calibration curve.

Thirteen calibration spikes were prepared in porcine plasma covering the concentration

range of 1 to 10,000 ng/mL. QC samples were prepared at concentrations of 15, 150, and 1500

ng/mL and had been stored at -80°C for 12 months. Results were then viewed in the Quan

Browser portion of the Xcalibur software. Calibration curves exhibited a correlation coefficient

($r2$) exceeding 0.999 across the entire concentration range with almost all of the calibrators

differing from their nominal value by less than 5%. QC samples at were within 2 to 8 % of their nominal values.

<u>Pharmacokinetic modeling and statistical analysis</u>

Plasma drug concentrations at respective time points were used to construct a time versus concentration curve for each pig. Noncompartmental pharmacokinetic (PK) analysis was applied to calculate the following clinically relevant parameters: area under curve time 0 to last time point (AUC_{0-last}), area under curve time 0 extrapolated to infinity ($AUC_{0-\infty}$), maximum concentration (C_{max}), time to maximum concentration (T_{max}), terminal half-life ($T_{1/2\lambda}$), terminal slope (λ_z), clearance per fraction of dose absorbed (Cl/F), volume of distribution per fraction of dose absorbed (Vz/F). PK modeling was conducted using WinNonlin® software (WinNonlin 5.2, Pharsight Corporation, Mountain View, CA).

Mixed model analysis of variance (ANOVA) was performed on PK parameters. Treatment was the fixed effect. The PK parameter of interest was the response variable. All PK parameters were treated as continuous, except T_{max} was treated as a class variable. Pairwise comparison of PK parameter and differences due to treatment were determined using Tukey's honest significant difference test (Tukey's HSD). Statistical significance was set a priori at values of $p \leq 0.05$. Statistical analysis was conducted using SAS software (version 9.4, SAS Institute, Cary, NC).

Results

<u>Routine diagnostics</u>

Prior to allotment, all pigs were naïve for PRRSv. Control pigs remained PRRSv naïve throughout the study. All pigs had evidence of the infection or vaccination that was expected for

their respective group and there was no evidence of cross-contamination among groups. Routine diagnostic and viral characterization results are summarized in Table 3.

Pharmacokinetics

Concentration versus time curves for mean plasma concentrations of desfuroylceftiofuracetamide (DCA) in the Control group, Vx group, Challenge group, and Vx+Challenge group are displayed in Figure 1. Means and ranges of evaluated PK parameters with pairwise comparisons are summarized in Table 4. Control group, Vx group, and Vx+Challenge group had no significant difference on any evaluated PK parameter.

The area under the curve of desfuroylceftiofuracetamide time zero to last time point is significantly lower in the Challenge group when compared to the other three groups. When considering $AUC_{0-\infty}$ the Challenge only group is significantly lower than the Vx group and the Vx+Challenge group, but the Challenge and Control groups are not significantly different. Percent extrapolated for $AUC_{0-\infty}$ is less than 4% for each group.

Clearance per fraction of dose absorbed (Cl/F) and volume of distribution per fraction dose absorbed (Vz/F) are significantly higher in the Challenge group when compare to any of the other three groups other three groups. The Vx+Challenge group had a significantly higher terminal slope (λ_z) than the Challenge group. C_{max} was not significantly different among any group. There is a numerical appreciation of the Vx+ Challenge group having a higher C_{max} than the Challenge group (Tukey's p=0.0995). T_{max} was significantly later in the Vx group when compared to the Challenge group.

Discussion

The first objective was to determine if vaccination with PRRS MLV impacted pharmacokinetics of CCFA. This study demonstrated that PRRS MLV does not impact the

pharmacokinetic parameters of CCFA compared to control animals that are not vaccinated or challenged. The second objective was to determine if PRRS MLV vaccination preserved pharmacokinetics of CCFA in the face of PRRSv challenge. This study determined vaccination with PRRS MLV preserved pharmacokinetic parameters in face of a wild type challenge.

Comparing control animals to those receiving only the PRRS MLV, no differences were observed. Previous work has demonstrated the PRRS MLV (Ingelvac MLV®) used is for study will cause virema starting around three days after inoculation with peak virema occurring seven to fifteen days after inoculation (Johnson et al., 2004). The present work targeted peak virema of PRRS MLV by having a PK assessment of 240 hours duration, which commenced five days after vaccination (Vx group). Viremia was confirmed in the respective group by having all animals test PRRSv positive by routine PCR five after inoculation. Likewise, PRRS MLV *in vivo* replication in lung or tonsil tissue was demonstrated with the PRRS MLV specific RT-PCR used on tissues collected at necropsy. Despite viremia and *in vivo* replication with the PRRS MLV, when compared to control animals, there were no differences in the evaluated pharmacokinetic parameters of CCFA.

Comparing control animals to those receiving a PRRSv wild-type challenge, CCFA in challenged animals had a higher Cl/F, higher Vz/F, and lower AUC_{0-last}. The PK assessment had a duration of 240 hours, which commenced five days after challenging the animals (Challenge group). This timing targeted peak virema of the wild type virus, VR-2385, based on previous literature (Johnson et al., 2004). Tantituvanont et al. (2008) found similar results with PRRSv challenged pigs and the pharmacokinetics of a shorter acting ceftiofur preparation, ceftiofur hydrochloride (HCl). Similar to observations in the present study with CCFA, ceftiofur HCl in PRRSv challenged pigs was reported to have a higher Cl/F, higher Vz/F and lower AUC.

Additionally, ceftiofur HCl in PRRSv infected animals was reported to have a lower C_{max}, shorter mean residence time, shorter half-life, and an increased terminal slope.

Comparing control animals to those vaccinated with the PRRS MLV prior to a PRRSv wild-type challenge, no differences were observed. Pigs had been vaccinated twenty-eight days before receiving a wild-type PRRSv challenge, and five days after challenge the pigs underwent the PK assessment for CCFA (Vx+Challenge group). This timing was aimed at achieving high levels of humoral and cellular immunity at time of challenge (Li et al., 2014). The timing also targeted peak viremia for the wild type virus at PK assessment (Johnson et al., 2004). At time of challenge, all animals of the respective group were positive for antibody by ELISA. Protective nature of the measured antibody was not determined. However, presence of antibody demonstrated that all pigs of the respective group had an immune response after vaccination. Furthermore, all animals of the respective group had wild-type PRRSv infection in lung or tonsil tissue demonstrated with the PRRSv VR-2385 specific RT-PCR used on tissues collected at necropsy, so the effect was not a consequence of preventing wild-type infection with vaccine. Despite the challenge and infection with the wild-type PRRSv, pigs that had been previously vaccinated had no differences detected in the evaluated pharmacokinetic parameters of CCFA when compared to control animals.

Other work has shown disease challenge in swine changes the pharmacokinetics of intravenous (IV) enrofloxacin, IV trimethoprim sulfadimethoxine, IV trimethoprim sulfamethoxazole, oral oxytetracycline, IV oxytetracycline, IM penicillin G, IV ampicillin, IV sulfadimidine, IV danofloxacin, intramuscular (IM) amoxicillin, and oral amoxicillin (Post et al., 2002; Post et al., 2003; Mengelers et al., 1995; Pijpers et al., 1991; Pijpers et al., 1990; Zeng and Fung, 1990, Yuan et al., 1997; Lindecrona et al., 2000; Agerso et al., 2000; Jensen et al., 2004;).

However, there is evidence that disease does not always change the pharmacokinetics of IV florfenicol, IM florfenicol, oral florfenicol, IV sulfadimethoxine, IV sulfamethoxazole, and IM ampicillin (Liu et al., 2003; Mengelers et al., 1995; Yuan et al., 1997). Studies observing changes in pharmacokinetics shared a common theme of changes in volume of distribution, clearance, or both. The pharmacokinetic changes seen in the present study are suspected to be from a combination of impacts on volume of distribution, clearance, and absorption (F, fraction of dose absorbed, bioavailability from injection site). For absorption to be determined, an IV pharmacokinetic model must be established and referenced for comparison. In this study absorption could not be determined, because there were not duplicate treatment groups receiving an IV dose of CCFA. Absorption rate and extent of CCFA from the IM injection site remains unknown. With non-intravenous pharmacokinetics, the volume of distribution and clearance remain dependent upon absorption. There is reason to believe that a PRRSv infection could impact absorption, volume of distribution, and clearance. Others have suggested that disease may influence volume of distribution and clearance by altering capillary permeability, hepatic function, renal function, and plasma proteins available for drug binding (Riviere, 2009; Tantituvanont et al. 2008). It also seems reasonable that an infection could change typical physiologic pH of the vasculature, which could potentially influence both absorption and protein binding.

The present work found no differences in C_{max} among any groups, and $T_{1/2\lambda}$ was higher in the pigs only receiving the wild-type PRRSv challenge when compared to pigs vaccinated prior to wild-type PRRSv challenge. It is well accepted that time above bacterial minimum inhibitory concentration (MIC) is a desired way to estimate effectiveness of β-lactam antibiotics (Papich, 2014). Considering the C_{max} and $T_{1/2\lambda}$ of the present work, it would be intuitive to suggest that

CCFA efficacy does not change in PRRSv challenged pigs regardless of vaccination status. However, a close examination of the concentration versus time curve should not be dismissed (Figure1). Consider the time each groups falls below 2μg/mL. The curve shows pigs receiving only the wild-type challenge fall below 2μg/mL approximately 24hours before any of the other groups. The Clinical and Laboratory Standards Institute (CLSI) has an established ceftiofur breakpoint, recognizing ceftiofur susceptibility at an MIC ≤2μg/mL for four organisms involved with swine respiratory disease, *Actinobacillus pleuropneumoniae, Pasteurella multocida, Salmonella choleraesuis,* and *Streptococcus suis* (CLSI, 2013). It is acknowledged that the breakpoint is based on the parent compound of ceftiofur, the main metabolite in pigs is desfurolyceftiofur, and the compound evaluated in the present study is a stable derivative representing ceftiofur metabolites. It is also acknowledged that using breakpoint criteria and a concentration versus time curve cannot be directly correlated with antimicrobial efficacy. However, present observations would suggest PRRS MLV vaccination has the potential to preserve time-dependent CCFA efficacy in pigs faced with PRRSv wild-type challenge.

Observations of the present study suggest that PRRS MLV does not change the pharmacokinetics of CCFA, PRRSv wild-type infection does change the pharmacokinetics of CCFA, and previous vaccination has the potential to preserve the pharmacokinetics of CCFA in face of a wild-type PRRSv challenge. The present study did not evaluate antimicrobial or vaccine efficacy. Future work could focus on understanding possible bioavailability changes. Additionally, measurements of vascular pH and plasma proteins could possible help suggest reasons for observed changes.

Acknowledgments

CHAPTER 4

POLYMERASE CHAIN REACTION TEST DEVELOPMENT AND APPLICATION FOR
VIRAL DETECTION, SEQUENCING AND QUALITY CONTROL IN A STUDY
DETERMINING VACCINATION MITIGATES THE IMPACT PRRSV INFECTION HAS ON
THE PHARMACOKINETICS OF CEFTIOFUR CRYSTALLINE FREE ACID IN PIGS

Introduction

The original research outlined in Chapter 3 used PRRS MLV vaccination and PRRSv
VR-2385 wild-type challenge. Meaningful pharmacokinetic results were contingent on high
confidence in the vaccination or wild-type infection status of the groups. Routine PRRSv PCR
assays are unable to differentiate viral strains. The development of a strain-specific PRRSv RT-
PCR, was needed to check and confirm the status of the study groups. This strain-specific
PRRSv RT-PCR was able to individually differentiate and quantify PRRS MLV strain and
PRRSv VR-2385 wild-type strain. In development and execution of strain specific RT-PCR,
viral sequencing was performed on samples. Sequencing was performed as a means to gain
better understand the genetic behavior of the virus in live animals. Sequencing also served as
quality assurance for the RT-PCR.

In addition to sequencing, lung histopathology and PRRSv immunohistochemistry (IHC)
on lung and tonsil tissue were performed as part of the research outlined in Chapter 3. In effort to
remain concise for publication, this information was not included in the journal submission
describing PK changes. This chapter includes the information regarding PRRSv sequencing,
histopathology scoring of lung tissue, and PRRSv IHC on lung and tonsil tissue.

PRRSv Sequencing

Sequencing was performed on select lung and tonsil samples to confirm validity of the
strain specific PCRs. Sequence data were assembled and analyzed using the DNAStar Lasergene

11 Core Suite. Aliquots of the specific VR-2385 inoculum and Ingelvac® MLV vaccine were used for quality assurance of the strain specific RT-PRCs and sequence referencing. All lung samples from the Control Group tested negative for PRRSV by all strain specific RT-PCRs.

For the Vx Goup, 8 out of 10 lung samples tested positive by MLV-specific ORF5 and nsp2 RT-PCRs. The PCR products were attempted for sequencing. The ORF5 PCR products from 8 lung samples were successfully sequenced and confirmed as Ingelvac® MLV. Attempts to sequence the nsp2 region of MLV virus detected in two lung samples were unsuccessful. However, the nsp2 region of MLV virus detected in the other six lung samples was successfully sequenced and confirmed as Ingelvac® MLV. For the two lung samples that were negative by MLV-specific ORF5 and nsp2 RT-PCRs, tonsils from the two pigs were tested by these MLV-specific RT-PCRs. The tonsils samples were positive by MLV-specific ORF5 and nsp2 RT-PCRs. The ORF5 and nsp2 regions of virus detected in one tonsil sample were successfully sequenced and confirmed as Ingelvac® MLV. But attempts to sequence the other tonsil sample were unsuccessful. None of the 10 lungs samples and 2 tonsil samples were positive by VR2385-specific ORF5 or nsp2 RT-PCRs.

For the Challenge Group, 10 out of 10 lung samples were positive by VR-2385-specific ORF5 and nsp2 RT-PCRs. The PCR products were successfully sequenced and confirmed as VR-2385 virus, except the nsp2 region of virus detected in one lung was unsuccessful in sequencing. All of the 10 lung samples were negative by MLV-specific ORF5 and nsp2 PCRs.

For the Vx+Challenge Group, only one lung sample was positive for both MLV and VR-2385 concurrently. Seven lung samples were positive for VR-2385 but negative for MLV. Sequencing confirmed the identities of PCR products. Two lung samples were negative for both VR-2385 and MLV. In order to determine if tonsils from this group of pigs contained both VR-

2385 and MLV, the 10 tonsil samples were tested by MLV-specific ORF5 and nsp2 RT-PCRs and VR-2385-specific ORF5 and nsp2 RT-PCRs. All of the 10 tonsils were positive for both Ingelvac® MLV and VR2-385 viruses and confirmed by sequencing.

The MLV viruses recovered from Vx and Vx+ Challenge groups shared 99.1-100% ORF5 nucleotide identities to each other and to the Ingelvac® MLV vaccine virus that was used to vaccinate pigs. Point mutations were observed at five positions of ORF5 and no insertions or deletions were observed. The MLV viruses recovered from the Vx and Vx+Challenge groups shared 99.2-100% nsp2 nucleotide identities to each other and 99.8-100% nsp2 nucleotide identities to the Ingelvac® MLV vaccine virus that was used to vaccinate pigs. Point mutations were observed at 5 positions of nsp2. Interestingly, a 9-nucleotide deletion 5'GTTCTGGGA3' at nucleotide positions 3118-3126 was observed in nsp2 region of MLV viruses recovered from three pigs in the Vx groups and three pigs in the Vx+Challenge group. The biological significance of this 9-nucleotide deletion was not determined.

The VR-2385 viruses recovered from Challenge and Vx+Challenge groups shared 99.8-100% ORF5 nucleotide identities to each other and to the VR-2385 PRRSV that was used to challenge pigs. Point mutations were observed at 2 positions of ORF5 and no insertions or deletions were observed. The VR-2385 viruses recovered from Challenge and Vx+Challenge groups shared 99.2-100% nsp2 nucleotide sequences to each other and 99.6-100% nsp2 nucleotide identities to the VR-2385 PRRSV that was used to challenge pigs. Point mutations were observed at six positions of nsp2 and no insertions or deletions were observed.

Histopathology & PRRSv IHC

Lung and tonsil tissues were collected at necropsy and placed in 10% buffered neutral formalin. After 24 hours of formalin fixation, tissues were transferred to 70% isopropyl alcohol

until hematoxylin and eosin staining and slide preparation was performed. Histopathology included scoring for interstitial pneumonia. The scores of 0, 1, 2, 3, 4, 5, and 6, corresponded to normal lung, mild multifocal interstitial pneumonia, mild diffuse pneumonia, moderate multifocal pneumonia, moderate diffuse pneumonia, severe multifocal pneumonia, and severe diffuse pneumonia, respectively. This interstitial pneumonia scoring system was based on previously established standards (Halbur et al., 1996). PRRSv immunohistochemistry (IHC) was performed on the formalin and alcohol fixed lung tissue. If lung tissue did not stain IHC positive, tonsil was then tested.

Statistical analysis was not performed on histopathology scoring or IHC data. Analysis was not performed, because the study design was not created to evaluate lung pathology. Sample size of treatment groups was constructed for pharmacokinetic evaluation, not lung pathology evaluation. The time lung samples were collected was not targeting peak virema. The use on an antibiotic could be a potential confounder.

Histopathology scoring results are summarized in Table 5. Histopathology scores averaged 2.25, 1.6, 3.9, and 3.0 for the Control, Vx, Challenge, and Vx+Challenge groups, respectively. The two groups receiving the wild-type challenge did have scores appreciably higher. Interestingly, the lung scoring averaged slightly better, scored lower, in the Vx Group when compared to the Control Group. There are several reasons why the average scores might not be exactly as expected. Scoring is subjective. Under the microscope, the person scoring is limited to make judgment on evaluation of very small portion of lung. Variation could occur in necropsy collection and sample preparation, as well.

PRRSV IHC results are summarized in Table 5. There were no positive samples in the Control Group. There were 3/10, 8/10, and 5/10 positives in the Vx, Challenge, and

Vx+Challenge Groups, respectively. In the Vx Group, one of the three positives only stained positive on tonsil. In the Challenge Group, five of the eight positives only stained positive on tonsil. In the Vx+Challenge group, four of the five positives only stained positive on tonsil. PRRSV IHC is often used for analytical specificity and not sensitivity. For an IHC to be positive, viral antigen needs to be present in the small section of lung being evaluated under the microscope. That viral antigen must have an epitope able to be bound by antibody in the IHC assay. The specificity and sensitivity was demonstrated in these results. The Control Group had no positives, confirmed my RT-PCR. The other groups had only a proportion of the group positive for PRRSv by IHC. However, all pigs in these groups were RT-PCR positive. This study was not constructed to evaluate or demonstrate PRRSv IHC capabilities. The data were used to help understand PRRSv infection, in contrast to all other information.

CHAPTER 5

SUMMARY AND CONCLUSIONS

PRRSv influence briefly summarized

The original work of this thesis demonstrated PRRSv infection changes the pharmacokinetics of CCFA. This is the first time this disease-antimicrobial interaction has been investigated. Furthermore, this original research investigated the potential impact of PRRS MLV vaccination. The vaccine did not change pharmacokinetics of CCFA, and when used as a vaccine, it could prevent PK changes seen in a PRRSv wild-type challenge. This is the first time a vaccine has been investigated with swine antimicrobial pharmacokinetics. There are three other swine studies that used PRRSv as an infection model (Day et al., 2015; Godoy et al., 2011; Tantituvanont et al., 2009). Two of the studies had a bacterial component (Day et al., 2015; Godoy et al., 2011). All three of the studies used a β-lactam antibiotics, and each study found disease changed pharmacokinetics.

There are two salient features that make PRRSv infection and vaccination ideal for investigation. First, PRRSv related disease has substantial impact on the swine population (Holtkamp et al. 2013). Second, the use of a viral challenge model allows disease-influenced pharmacokinetic changes to be better attributed to physiological changes and not presence of an organism. In some pharmacokinetic studies the use of an organism may be considered a confounder, because of the potential consumption, metabolism, or other alteration of the antimicrobial by target bacteria. An example would be pharmacokinetic changes of penicillin G in *S. suis* challenged pigs (Zeng and Fung, 1990); some of the pharmacokinetic changes could be a result of antimicrobial consumption by the *S. suis* organism. In the pure sense, it would be best

to know the pharmacokinetic changes that are a result of the body's physiologic change in a disease state. Viral and toxin challenge models avoid this potential confounder.

It is biologically intriguing that pharmacokinetic differences were observed in PRRSv wild-type challenged pigs and not pigs receiving PRRS MLV. It is noteworthy that previous PRRS MLV vaccination prevented pharmacokinetic changes observed in face of a PRRSv wild-type challenge. Ingelvac® PRRS MLV, used in the present research, has been available as a commercial vaccine since 1996, and it is simply an attenuated VR-2332 field virus that was passaged in monkey kidney cells (Kim et al., 2008). There is a vast amount of research evaluating PRRSv infection, and there is a decent body research that evaluates PRRS MLV. One study found significantly less severe lung lesions in pigs inoculated with PRRS MLV when compared to pigs inoculated with VR-2385, the challenge strain used in the present research of this thesis (Opriessnig, et al., 2002). However, they did find some lesions in the group only receiving PRRS MLV. Another study measured immune responses in pigs vaccinated with PRRS MLV prior to challenge with homologous or heterologous PRRSv (Li et al., 2014). They found PRRS MLV vaccination induced neutralizing antibodies with partial protection against heterologous challenge, but they found cytokine expressions and T-cell subpopulations did not differ between homologous or heterologous challenge. These two studies are examples of some observed behavior of the PRRS MLV, yet there is much unknown.

There are several mechanisms by which PRRSv could be inhibiting a pig's ability to normally absorb, distribute, metabolize, or excrete a drug. PRRSv has the potential to influence many of the previously mentioned pharmacokinetic changing mechanisms, renal function, protein binding, vascular function, and many others. PRRSv has potential to cause vasculitis and necrosis in kidneys (Cooper et al., 1997). Considering protein binding, there are numerous

studies on PRRSv evaluating cytokine expression and immune cells response, all which have the potential to change protein binding in the blood (Parra et al., 2006; Gómez-Laguna et al., 2010; Silva-Campa 2009; Wongyanin et al., 2010). The generalized infection and inflammation caused by PRRSv could cause changes in vascular permeability and blood flow (Halbur et al., 1995; Halbur et al., 1996; Rossow, 1998). With the disease caused in lungs and possible kidneys, it is also reasonable to consider that blood pH could be another mechanistic change. There are several other mechanisms that could conceivably contribute to changes seen. Vaccination with PRRS MLV could partially or fully prevent specific changes.

In the present work of the thesis and with the bounds of the materials and methods, PRRS MLV vaccination does not appreciably change the way a pig's body handles CCFA. PRRSv challenge does change the way a pig's body handles CCFA. PRRS MLV vaccination prior to PRRSv challenge allows the pig's body to handle CCFA as if it were not challenged.

Future areas to investigate

To attempt to understand reasons behind the observed pharmacokinetic changes, future research could be based from altered pharmacokinetic parameters observed with this present study, Cl, Vd, and F. The faction of dose absorbed (F, bioavailability) was not measured in the present study because of the lack of IV treatment groups. Obtaining IV pharmacokinetic data to establishing bioavailability knowledge would help understanding. Measuring blood pH might be a means to understand bioavailability and drug distribution. As mentioned earlier, compound ionization is often dependent on pH, and ionization influences the ability for a compound to cross biological membranes, which is why measuring blood pH may be insightful. Evaluating tissue concentrations could help understand distribution. Measuring protein binding could help

elucidate distribution and clearance. Evaluating renal function and elimination would be methods for understanding clearance.

Clinical implications and final conclusions

Pharmacokinetic data are used to establish dosing regimens for efficacious treatment. Within food animals, pharmacokinetic data are also important to establish drug withdrawal times to avoid antimicrobial residues in the human food supply. Disease has the potential to change pharmacokinetics and subsequently change antimicrobial efficacy or withdrawal times. The specific drug and disease seem to be relevant as to whether pharmacokinetic changes will truly occur. However, the available information for swine suggests a majority of the time disease will change the pharmacokinetics of a given drug. When faced with using an antibiotic in clinical practice, a literature search should first be completed attempting to find if research has been done with the specific disease and antibiotic of interest. If there is not a match, utilization of the best available data should be implemented. After drug use, a case should be followed to note treatment success and failure. The information from research and diligent experience is what should be utilized to go forward with practicing antimicrobial usage. In clinical food animal practice, anytime a drug is being administered, the question should be asked, "Is this drug being given at the correct dose and route in order to reach the target site in the animal at a level that will be efficacious, yet not toxic or in residue violation?"

Figure 1. Plasma concentration versus time curves for mean plasma concentrations of desfuroylceftiofuracetamide.
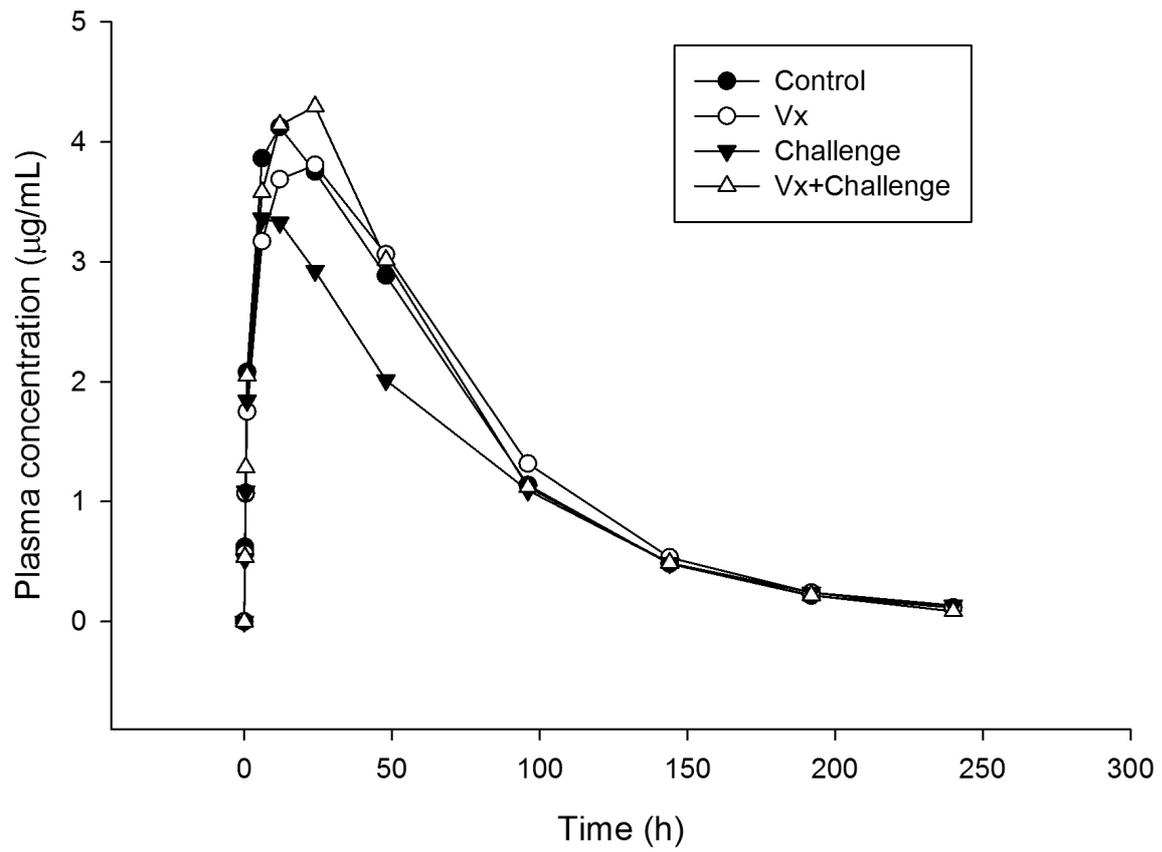
Table 1. Summary of studies evaluating disease influence on antimicrobial pharmacokinetics is swine (adapted from Day, 2014)

Disease	Drug Evaluated			PK Parameters Evaluated (Increase, Decrease)											Citation
Agent	Drug	Route	Dose (mg/kg)	AUC	C_{MAX}	MRT	T_{MAX}	$T_{1/2}$	Vz		CL		Elimination		
APP	Florfenicol	IV	20	NSD	NSD	NSD	NSD	NSD	V_{SS}	NSD	Cl	NSD	-----	-----	Liu, 2003
	Florfenicol	IM	20	NSD	NSD	NSD	NSD	NSD	V_{SS}	NSD	Cl/F	NSD	-----	-----	Liu, 2003
	Florfenicol	POg	20	NSD	NSD	NSD	NSD	NSD	V_{SS}	NSD	Cl/F	NSD	-----	-----	Liu, 2003
	Enrofloxacin	IV	5	NSD	-----	-----	-----	↓	V_{SS}	↑	Cl_{tot}	NSD	β	↑	Post, 2002
	Enro + dexamethasone	IV	5, 0.5	↓	-----	-----	-----	↓	V_{SS}	NSD	Cl_{tot}	↑	β	↑	Post, 2002
	Amoxicillin	IV	8.6	NSD	-----	NSD	-----	NSD	V_{ss}	NSD	Cl_{tot}	NSD	-----	-----	Agerso, 1998
	Sulfadimethoxine§	IV	25	NSD	-----	-----	-----	NSD	V_d	NSD	Cl_{tot}	NSD	-----	-----	Mengelers, 1995
	Sulfamethoxazole§	IV	25	NSD	-----	-----	-----	NSD	V_d	NSD	Cl_{tot}	NSD	-----	-----	Mengelers, 1995
	Trimethoprim†,§	IV	5	↓	-----	-----	-----	NSD	V_d	↑	Cl_{tot}	↑	-----	-----	Mengelers, 1995
	Trimethoprim‡,§	IV	5	↓	-----	-----	-----	NSD	V_d	↑	Cl_{tot}	↑	-----	-----	Mengelers, 1995
	Oxytetracycline§	POg	50	↑	↓	-----	↑	NSD	V_d	↑	Cl_{tot}	↓	β	↓	Pijpers, 1991
	Oxytetracycline§	IV	10	↑	-----	-----	-----	↓	$V_{dβ}$	↓	Cl_{tot}	↓	β	↑	Pijpers, 1990
	Oxytetracycline§	IV	50	NSD	-----	-----	-----	NSD	$V_{dβ}$	NSD	Cl_{tot}	NSD	β	↑	Pijpers, 1990
PRRSV	Ceftiofur hydrochloride	IM	3	↓	↓	↓	NSD	↓	Vz/F	↑	CL/F	↑	$λ_z$	↑	Tantituvanont, 2009
	Amoxicillin*	POa	9	↑	↑	NSD	-----	-----	-----	-----	Cl_p	NSD	-----	-----	Godoy et al., 2011
PRRSV + *Strep suis*	Ceftiofur hydrochloride	IM	5	↓	↓	NSD	NSD	NSD	Vz/F	↑	CL/F	↑	K_{el}	NSD	Day et al., 2015
Strep suis	Penicillin G	IM	15,000 iu	↓	↓	-----	NSD	NSD	V_d	↑	Cl_b	↑	K_{el}	NSD	Zeng & Fung, 1990
Strep suum	Ampicillin	IV	10	↓	-----	-----	-----	NSD	V_{ss}	↑	Cl_b	↑	K_{10}	↑	Yuan, 1997
	Ampicillin	IM	10	NSD	-----	-----	-----	NSD	V_{ss}	NSD	Cl_b	NSD	-----	-----	Yuan, 1997
	Sulfadimidine	IV	50	↑	-----	-----	-----	↑	V_{ss}	NSD	Cl_b	↓	K_{10}	NSD	Yuan, 1997
Salmonella typhimurium	Danofloxacin	IV	2.4	↑	-----	-----	-----	↑	V_{ss}	↓	Cl_b	↓	-----	-----	Lindecrona, 2000
	Amoxicillin	IM	15	NSD	NSD	↑	NSD	↑	V/F	↑	-----	-----	K_{el}	↓	Agerso, 2000
E. coli	Amoxicillin	POa	26-32	↓	↓	-----	↑	↑	-----	-----	-----	-----	K_{el}	↓	Jensen, 2006
	Amoxicillin	POg	20	↓	↓	-----	NSD	NSD	-----	-----	-----	-----	K_{el}	NSD	Jensen, 2004
	Enrofloxacin	IV	5	↑	-----	-----	-----	↑	$V_β$	NSD	Cl_b	↓	β	↓	Post, 2003
	Enro + dexamethasone§	IV	5, 0.5	NSD	-----	-----	-----	NSD	$V_β$	↑	Cl_b	NSD	β	NSD	Post, 2003
	Antipyrine§	POg	150	-----	-----	-----	-----	↑	V_d	↑	Cl_{tot}	-----	K_{el}	↓	Ladefoged, 1979#
	Trimethoprim§	POg	20	-----	-----	-----	-----	↑	V_d	↑	Cl_{tot}	-----	K_{el}	↓	Ladefoged, 1979#

NSD = No Significant Difference, ↑ = PK parameter increased, ↓ = PK parameter decreased, ---- = PK parameter not evaluated,

POg = *per os* by gavage, POa = *per os* by *ad libitum*

† In combination with 25 mg SDM/kg BW ‡ In combination with 25 mg SMX/kg BW

*Natural infection with the primary agent being PRRSV, and *P. multocida*, *B. bronchiseptica*, and *S. suis* as opportunistic pathogens.

§Disease challenged performed with toxin or LPS, not actual organism.

#Study did not statistically report

Table 2. Summary of study design events.

| Group (code) | No. of pigs | Event and study day | | | |
		Vaccination with Ingelvac® PRRS MLV	Challenge with PRRSv VR-2385	PK Assessment	Necropsy
Control (Control)	8	None	None	Days 0 to 9	Day 9
PRRS MLV (Vx)	10	Day -5	None	Days 0 to 9	Day 9
PRRSv Challenge (Challenge)	10	None	Day -5	Days 0 to 9	Day 9
PRRS MLV vaccination followed by Challenge (Vx+Challenge)	10	Day -33	Day -5	Days 0 to 9	Day 9

Table 3. Summary of PRRSv routine diagnostics and viral characterization

| Group | Study Day, test type, and frequency of positives | | | | | | |
| | Prior to allotment | | -5 | 0 | 9 | | |
	Routine PCR	ELISA	ELISA	Routine PCR	Routine PCR	MLV specific PCRs	VR-2385 specific PCRs
Control			NP	0/8	0/8	0/8	0/8
Vx			NP	10/10	9/10	10/10	0/10
Challenge	0/38	0/38	NP	10/10	10/10	0/10	10/10
Vx+ Challenge			10/10	9/10	8/10	10/10	10/10

Routine PRCs and antibody ELISAs were run on serum. MLV and VR-2385 specific PCRs were run on lung or tonsil. NP, not performed

Table 4. Plasma pharmacokinetic parameters (arithmetic mean and range) of ceftiofur and related metabolites amongst treatment groups. Values in a row with identical superscripts are not significantly different (Tukey's HSD, $P < 0.05$).

Parameter	Units	Control	Vx	Challenge	Vx+Challenge
AUC % extrapolated	%	2.20 (0.85-4.72)[a,b]	2.19 (0.76-4.45)[a,b]	3.87 (1.45-10.5)[a]	1.41 (0.32-2.70)[b]
$AUC_{0-\infty}$	h*µg/mL	334 (281-400)[a,b]	345 (303-415)[a]	281 (194-332)[b]	344 (286-418)[a]
AUC_{0-last}	h*µg/mL	327 (277-390)[a]	338 (297-411)[a]	270 (185-321)[b]	339 (281-410)[a]
Cl/F	mL/h/kg	15.2 (12.5-17.8)[a]	14.6 (12.1-16.5)[a]	18.2 (15.1-25.8)[b]	14.8 (12.0-17.5)[a]
C_{max}	µg/mL	4.30 (3.24-5.42)[a]	3.91 (2.82-6.13)[a]	3.68 (2.61-4.89)[a]	4.55 (3.52-5.61)[a]
$T_{1/2\lambda}$	h	41.7 (33.4-55.3)[a,b]	42.4 (29.9-53.0)[a,b]	50.9 (39.6-95.2)[a]	38.0 (29.4-43.9)[b]
λ_z	1/h	0.01692 (0.01-0.02)[a,b]	0.01695 (0.01-0.02)[a,b]	0.01459 (0.01-0.02)[a]	0.01859 (0.02-0.02)[b]
T_{max}	h	14.3 (6.00-24.0)[a,b]	24.0 (12.0-48.0)[a]	11.4 (6.00-24.0)[b]	19.8 (6.00-24.0)[a,b]
Vz/F	mL/kg	915 (689-1310)[a]	899 (604-1130)[a]	1330 (987-2140)[b]	804 (602-965)[a]

Table 5. Summary of histopathology and PRRSv IHC

Group	n	Histopathology: average lung score (range)	PRRSv IHC (proportion of positive animals, lung or tonsil)
Control	8	2.25 (1-5)	0/8
Vx	10	1.6 (0-3)	3/10
Challenge	10	3.9 (1-6)	8/10
Vx+Challenge	10	3.0 (1-5)	5/10

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