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Analysis of procaine penicillin G and ampicillin trihydrate residue depletion profiles in tissues and alternative matrices of cull sows

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Analysis of procaine penicillin G and ampicillin trihydrate residue depletion profiles in tissues and alternative matrices of cull sows

by

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DEDICATION

I would like to dedicate this thesis to my family. To my husband Alex, thank you for allowing me to pursue my dreams and offering support and encouragement along the way. To my mom Sheila, you have always believed in me and given me the guidance and love I needed to persevere. To my brother John, you have provided humility and a sense of duty. Finally I would like to dedicate this thesis to my dad Steve. You were always my loudest cheerleader, my biggest critic, and my greatest inspiration.

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NOMENCLATURE

AUC	Area Under the Curve, for a concentration vs. time plot
Cmax	Maximum Plasma Concentration
EPA	Environmental Protection Agency
FARAD	Food Animal Residue Avoidance Databank
FAST	Fast Antimicrobial Screen Test
FDA	Food and Drug Administration
FMIA	Federal Meat Inspection Act
FSIS	Food Safety and Inspection Service
GABA	Gamma-aminobutyric acid
HPLC	High Performance Liquid Chromatography
IM	Intramuscular
IPP	Inspection Program Personnel
KIS	Kidney Inhibition Swab
LCMS	Liquid Chromatography Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MRM	Multi-residue method
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NADA	New Animal Drug Application
NRP	National Residue Program

PBP	Penicillin-binding Protein
PHV	Public Health Veterinarian
PPB	Parts per Billion
PPG	Procaine Penicillin G
PPIA	Poultry Products Inspection Act
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
MIC	Minimum Inhibitory Concentration
MRL	Maximum Residue Level
NRC	National Research Council
SID	*Semel in die* ("once a day")
UPLC	Ultra-high performance liquid chromatography

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ABSTRACT

Beta-lactam antimicrobials have historically been used as a treatment for illness in sows. In 2011 the FSIS updated its residue screening technologies, resulting in an increase of penicillin G residues in cull sows at slaughter. Little information is available concerning tissue residue depletion of procaine penicillin G or ampicillin trihydrate in sows.

Original research was performed to characterize the tissue residue depletion profiles and calculate withdrawal periods of procaine penicillin G and ampicillin trihydrate in sows using LC-MS/MS analysis. Penicillin G residues persist in kidneys, skeletal muscle, and injection sites for extended periods following treatment. The residues persist longer at higher doses, and a withdrawal period of 30 days is needed to ensure penicillin residue depletion in the kidney at 15,000 IU/lb IM SID for three consecutive days. There is no evidence for environmental transfer of penicillin G residues from treated sows to untreated sows. Ampicillin trihydrate is metabolized quickly by the sow, with residues only detected in the kidney at one day following treatment. Residues persisted at high concentrations at the injection sites for 40 days following treatment at the conclusion of the study. Injection site withdrawal times were estimated at 89 days for a 12 mg/kg IM SID dose for three consecutive days. Urine and plasma are potential ante-mortem screening matrices for penicillin G or ampicillin. Environmental samples and oral fluids are not good samples for beta-lactam residue analysis. Charm MRL™ test kits are a fairly sensitive and specific urine screening method for residue detection in urine.

Further research is needed to determine withdrawal times of ampicillin trihydrate in sows in edible tissues, and to evaluate the efficacy of alternative screening methods.

Available research describes a metabolism of procaine penicillin G and ampicillin trihydrate based on absorption, which is rapid once drug enters circulation. Original research supports this, but shows an extended residue of both drugs at injection sites following treatment. This information may be applied to establish recommendations for withdrawal times on extra-label beta-lactams and used clinically as a guide for the protection of meat from violative residues entering the consumer supply.

CHAPTER I

INTRODUCTION

Thesis organization

This thesis consists of four chapters. Chapter 1 includes thesis organization and existing knowledge of procaine penicillin G and ampicillin trihydrate drug properties, indications, pharmacokinetics, and proposed withdrawal times. It also focuses on regulation of drug residues, screening and confirmatory testing methodologies, and implications of violative drug residues. Chapter 2 is titled "Procaine penicillin G residue depletion in tissues of cull sows with correlation to residue concentrations in plasma, urine, and environmental samples" and will be submitted to the *Journal of Veterinary Pharmacology and Therapeutics* for peer review. Chapter 3 is titled "Tissue residue depletion profile of ampicillin trihydrate in cull sows and comparison with plasma, urine, and oral fluid residues" and will be submitted to the *Journal of Veterinary Pharmacology and Therapeutics* for peer review. Chapter 4 is a summary and conclusion of the previous chapters.

Beta-lactam antimicrobials

Beta-lactam antimicrobials are so named due to their chemical structure. The drugs all contain a beta-lactam ring that serves as a structural analog to the peptide bridge formed in bacterial cell wall synthesis. Thus, the beta-lactam class of drug exerts its bactericidal effects by interfering with cell well synthesis and destroying the integrity of the cell wall. The beta-lactam binds to enzymes called penicillin-binding-proteins (PBP's), which are normally used to complete cell wall synthesis in bacteria. The incorporation of a beta-lactam into the cell wall by

the PBP during the cross-linking of peptidoglycans results in a defective cell wall. The bacterium is then destroyed usually by lysis and osmotic instability (Papich and Riviere, 2009).

Effectiveness of beta-lactams across different bacterial spectra may vary based on the different types of PBP's present in these bacterial species. A bacterium may have 2-8 different PBP's in the cell wall. However, some bacteria have evolved to have a decreased sensitivity of these PBP's to beta-lactams, decreasing the effectiveness of this class of drug.

Beta-lactams enter the bacteria through porin proteins. Gram positive bacteria are more easily accessed than gram negative bacteria since gram negative bacteria have a thick outer membrane, and the porins are more difficult to penetrate (Papich and Riviere, 2009).

Beta-lactams are time-dependent antimicrobials. This is due to their slow bactericidal effect over time. For effectiveness of the drug to take effect, it must maintain levels in the target tissue above the minimum inhibitory concentration (MIC) for an extended period of time to obtain clinical success. Gram positive bacteria generally have a lower MIC than gram negative bacteria, which allows for a longer dosing interval when treating gram positive infection. Beta-lactam antibiotics must remain above the MIC for at least 50% of the dosing interval (Papich and Riviere, 2009).

Penicillins are active against non-beta-lactamase producing gram positive bacteria including many *Streptococci* spp. and *Staphylococci* spp. They are also active against select gram-negative aerobes including *Pasteurella multocida*, *Listeria monocytogenes*, and *Trueperella*. Some anaerobes including *Fusobacterium*, *Bacteroides*, and *Clostridium* are also susceptible. Penicillins are very effective against spirochete organisms. Aminopenicillins, including ampicillin, have the same spectrum of activity as penicillin G. However, they can

more readily enter the outer membrane of gram negative bacteria, and more quickly move through their porin proteins. (Papich and Riviere, 2009).

Ampicillin is used as a treatment for swine respiratory disease as well as other swine diseases. Salmon et al. (1995) performed MIC determinations of a variety of disease-causing bacteria for ampicillin in swine. Ampicillin was particularly effective against *Pasteurella multocida*, beta-hemolytic *Streptococcus* spp., and *Streptococcus suis*. It was moderately effective against *Salmonella typhimurium*. Contrary to previous MIC data, ampicillin had low efficacy against *E. coli*, *Salmonella cholerasuis*, and *Actinobacillus pleuropneumoniae* (Salmon et al., 1995).

A compilation of microbial susceptibility data from 2014 bacterial isolates at the Iowa State University Veterinary Diagnostic lab reports the percent susceptibility for a variety of antibiotics and bacterial species. For porcine pathogens, ampicillin had a greater percent susceptibility across all pathogens than penicillin. Susceptibility to ampicillin was reported in percentages for the following organisms: *A. suis* (96%), *A. pleuropneumoniae* (91%), *B. bronchiseptica* (6%), *E. coli* (34%), hemolytic *E. coli* (18%), *E. rhusiopathiae* (94%), *H. parasuis* (99%), *P. multocida* Type A (99%), *P. multocida* Type D (100%), *S. suis* (96%), and *Salmonella* spp. (65%). The following values were the reported susceptibilities for penicillin for porcine pathogens: *A. suis* (2%), *A. pleuropneumoniae* (19%), *B. bronchiseptica* (0%), *E. coli* (0%), hemolytic *E. coli* (0%), *E. rhusiopathiae* (89%), *H. parasuis* (31%), *P. multocida* Type A (92%), *P. multocida* Type D (93%), *S. suis* (78%), and *Salmonella* spp. (0%) (Iowa State University, 2014).

There are very few adverse effects associated with penicillins. The most common adverse effect noted is associated with immune-mediated reactions or anaphylaxis. In humans,

15% of the population experience some sort of allergy to penicillin. This allergy has been described in animal species as well. Most allergic reactions to penicillin in food are mild and consist of mainly dermatologic symptoms (Sundlof, 1989). They are also very uncommon. In a few rare cases, more serious anaphylactic reactions may occur (Lindemayr et al., 1981; Wicker et al., 1969). A second adverse effect is associated with the central nervous system. When administered at high concentrations, penicillins may act as GABA inhibitors. This inhibition presents as seizures and excitement. Procaine may also cause these clinical signs, which has been described in horses (Papich and Riviere, 2009).

Rather than measuring an amount of penicillin G by mass, it is instead described in terms of unitage to describe biological effect. The unit of penicillin is equal to the amount of specific activity in 0.6 ug of sodium penicillin. Penicillin G dosing also varies depending on the species to be treated, the disease targeted, and the formulation of penicillin G to be injected. Procaine penicillin G is typically given from 22,000-70,000 units of procaine penicillin G per kilogram of bodyweight once or twice daily. This is injected intramuscularly or subcutaneously. Some infections, such as those due to *Streptococcus* spp., may require a lower dose of procaine penicillin G. Others such as *Trueperella* may require much higher doses, even as high as 100,000 units per kilogram. The labelled doses for use of procaine penicillin G in food animals are much lower than what is indicated for treatment of infection and thus, extended withdrawal times are needed (Papich and Riviere, 2009).

There are multiple available procaine penicillin G products currently distributed over the counter. It is available in a liquid aqueous suspension. The suspension contains a concentration of 300,000 units of penicillin G per milliliter. It is indicated for intramuscular injection. In swine, the dose on the label is 3,000 units per pound of body weight per day. This equates to

6,600 units per kilogram bodyweight. The indications for treatment with procaine penicillin G in swine only include the treatment for erysipelas caused by *Erysipelothrix rhusiopathiae*. Treatment can be given for up to four days and should be administered for 24-48 hours after the cessation of clinical signs. Procaine penicillin G is also labeled for use in cattle, horses, and sheep. In cattle and sheep it has a label for the treatment of bacterial pneumonia caused by *Pasteurella multocida*, and in horses it is labeled for the treatment of strangles caused by *Streptococcus equi* (NADA 065-010).

According to Payne et al. (2006), penicillin is one of the most common residues that are detected in tissues during plant screenings. Questions about its extra-label use and withdrawal times are asked more frequently than any other antibiotic (Payne et al, 2006). In the uncooked edible tissues of swine, there is a zero tolerance for procaine penicillin G residues. This is the same in chickens, pheasants, quail, eggs, and sheep. Cattle have a 50 ppb tolerance in uncooked edible tissues, and turkeys have an established 10 ppb tolerance (NADA 065-010). For the labeled dose and duration, a withdrawal period of 7 days is given for swine (NADA 065-010). The injection volume of procaine penicillin G should not exceed 10 mL in one location. Additionally, consecutive injections should be given in different locations.

The only ampicillin trihydrate product currently marketed is Polyflex® by Boehringer Ingelheim Vetmedica, Inc. The Food and Drug Administration's (FDA) New Animal Drug Application (NADA) number for Polyflex is 055-030. It is labeled in domestic cats, dogs, and cattle. It can be administered via intramuscular or subcutaneous injection in cats and dogs or intramuscular injection in cattle. There is an established tolerance for ampicillin trihydrate in the United States. In the uncooked edible tissues of swine, cattle, and in milk, the established tolerance for ampicillin residues is 0.01 ppm (NADA 055-030).

The labeled dose in cattle is 2-5 mg/lb SID IM. It is indicated for the treatment of organisms causing respiratory tract infection that are susceptible to ampicillin and bacterial pneumonias caused by *Aerobacter* spp., *Streptococcus* spp, *Staphylococcus* spp., *Klebsiella* spp., *Pasteurella multocida*, and *E.coli*. The recommended treatment timeline for all animals is 3 days with extended treatment from 48 to 72 hours after dissolution of fever or clinical signs. In cattle, animals can be treated for a maximum of seven days and require a 48 hour withdrawal on milk or a 6 day withdrawal for meat (NADA 055-030).

Penicillin and ampicillin pharmacokinetics and residues

Injection of an aqueous suspension of the penicillins via a subcutaneous or intramuscular route results in rapid absorption of the drug. This is particularly true of the sodium salt formulations. The trihydrate formulation of ampicillin makes more a more stable molecule and also prolongs the absorption from the injection site after administration of the drug. This is primarily to create a longer-acting drug, achieving extending plasma concentrations. Penicillin G also has several formulations to promote a longer absorption. Procaine penicillin promotes longer absorption from the injection site, and thus prolonged plasma concentrations. The half-life of procaine penicillin G is longer than the aqueous salt formulation, potassium penicillin, but 24-hour plasma concentrations are relatively similar. A third penicillin formulation, benzathine penicillin, exhibits a much longer absorption period due to insolubility, which resulted in a lower, but extended plasma concentration when compared to potassium penicillin or procaine penicillin (Papich and Riviere, 2009).

Penicillins are eliminated by renal excretion through a tubular secretion mechanism and can be found in high concentrations in the urine. Both slow release formulations procaine

penicillin and ampicillin trihydrate exhibit flip-flop kinetics, which means that their rate of elimination depends on the rate of their absorption from the injection site. These two formulations have an extended half-life. Procaine penicillin may have an elimination half-life of greater than 20 hours, which also prolongs its antibiotic effect to greater than 24 hours. In cattle, a half-life of 6.7 hours has been reported for ampicillin trihydrate (Papich and Riviere, 2009).

The volume of distribution of penicillins is moderate. They diffuse into extracellular fluid fairly easily, depending on the percentage of protein-binding which may range from 30-60%. Penicillin is in an ionized form in the plasma. It can reach necessary concentrations for antimicrobial activity in the kidney, synovial fluid, lung, skin, liver, and other soft tissues. There is very little penetration of the blood brain barrier, but it may do so at very high concentrations or in a state of blood-brain-barrier compromise due to a diseased state (Papich and Riviere).

A 2013 Study by Smith et al. was conducted to determine the penicillin G depletion in sows. An extra-label dose of 33,000 IU/kg procaine penicillin G was administered to 126 heavy sows intramuscularly once a day for three consecutive days. This dose is five times the approved dose for penicillin swine. Sows were euthanized at multiple time periods over 39 days following the final day of dosing. Kidney and skeletal muscle tissues were collected and analyzed for penicillin G residues by UPLC-MS/MS (Smith et al, 2013).

Their study determined that penicillin G residues are depleted much faster from skeletal muscle tissues than kidney tissues. A calculated extended withdrawal time for skeletal muscle tissues was determined to be 13 days. The extended withdrawal time to ensure penicillin G residue depletion from the kidney was determined to be 52 days. Withdrawal times were calculated to ensure residue depletion in 99% of animals with a 95% confidence interval, using a 25 ppb action level for penicillin G residues in tissues at slaughter (Smith et al., 2013).

FARAD recommendations at that time were a 15 day withdrawal for penicillin G in swine, which would be sufficient for skeletal muscle depletion, but not kidneys. It was the conclusion of the authors that for penicillin G to remain a useful veterinary drug for treatment in heavy sows or swine, kidney tissues from treated animals should be considered not fit for human consumption (Smith et al, 2013).

Shelver et al. (2014) examined injection site residues in the sows of the Smith et al. (2013) study. Injection sites were analyzed for penicillin G residues by Charm KIS™ testing and by LC-MS/MS methods. On KIS™ tests, injection site positive tests were fairly similar to kidney positive tests. At 39 days post-treatment 3 out of 18 sows sampled had positive KIS™ injection sites. When compared to LC-MS/MS analysis, all of the positive KIS™ test samples were also positive on LC-MS/MS with concentrations ranging from 3.7-1254 ng/g. Of the injection site KIS™ tests, there were 5 false positives and 13 false negatives when compared to the reference standard LC-MS/MS. This may indicate inconsistency and difficulty with injection site analysis. One theory for the discrepancies could be due to high variability of penicillin G concentration at the injection site itself. Fat can also interfere with KIS™ testing, which may be a factor in the injection site samples. Previous studies have noted issues with intramuscular injection versus intermuscular injection and how that may affect penicillin G absorption at the injection site. An intermuscular injection resulted in slower absorption when compared to and intramuscular injection. Difficulty defining and obtaining injection sites at the time of tissue collection may also play a role in the wide variability of results (Shelver et al., 2014).

A study comparing subcutaneous injections of procaine penicillin G to intramuscular injections of procaine penicillin G was performed by Ranheim et al. (2002) in piglets. A single injection at a dose of 100,000 IU/kg was administered and plasma concentration levels were

serially collected. Both the half-life and then AUC were greater for subcutaneous injections of procaine penicillin G than the intramuscular injections. For the intramuscular and subcutaneous injection, plasma concentrations of penicillin G remained above the MIC for *Streptococcus suis* for 1-2 days following treatment (Ranheim et al., 2002).

Apley et al. (2009) compared the tissue residue profile of procaine penicillin G in cull sows administered at 33,000 IU/kg in cull sows by conventional injection versus a needle free injection. In that study, 20 sows were administered a single dose of PPG by conventional injection and 20 were administered PPG by needle-free injection. No more than 10 mL of penicillin was injected at a single location for the conventional injection method, and a volume of 5 mL was used for the needle-free injection. Plasma samples were collected for pharmacokinetic analysis and tissue samples were collected at 2, 4, 6, and 8 days following treatment. Analysis of samples was performed with UPLC-MS/MS, an ultra high-pressure LC-MS/MS analytical method, and histological examination of the injection sites (Apley et al., 2009). Although an ideal withdrawal study would have a large number of animals and have no detectable residues in all samples by the final study time point, this was not achieved with this study. This study showed that 8 days is not sufficient for procaine penicillin G residue depletion in the kidney, liver, muscle, or injection site for either method of administration (Apley et al., 2009). A comparison of the two injection methods showed that conventional injection results in a slower absorption, resulting in a longer half-life and longer Tmax. Results of the tissue analysis demonstrated a detectable penicillin residue in kidney tissues of 2 out of 5 sows at 8 days following treatment. Withdrawal calculations were performed on the kidney concentration values and a time of 28 days following treatment was determined to be sufficient for a 95%

certainty that 99% of the population would not have a positive residue for penicillin G (Apley et al., 2009).

Apley et al. also examined injection site effects. Injection site concentrations were compared to kidney concentrations of penicillin G residue. In the needle-free injection group, injection site penicillin concentrations were closely correlated with kidney tissue penicillin concentrations. However, the conventional injection group had much higher penicillin G concentrations at the injection site than in the kidney (Apley et al 2009). Histologic examination of the injection site was conducted to determine the extent of tissue alteration between the two methods. The conventional injection method had significantly fewer histologic alterations that the needle-free injection, which was somewhat surprising due to the higher concentrations of penicillin G detected at the injection site in the conventional injection method (Apley et al., 2009).

A 1998 study by Korsrud et al. administered intramuscular procaine penicillin G at extra-label doses to swine. They administered penicillin once daily for five days in a row at 15,000 U/kg or 66,000 IU/kg. The tissue residue results from this study resulted in the Canadian government's determination of an 8 day withdrawal for the 15,000 u/kg dose and a 15 day withdrawal for the 66,000 U/kg dose (Payne et al., 2006). They recommended using an 18 gauge, 4 cm needle with a maximum volume of 11 ml per injection site for best injection technique (Korsrud et al., 1998). These recommendations were supported by FARAD.

A study to examine ampicillin pharmacokinetics was performed by Martinez et al (2001). A single injection of ampicillin trihydrate in finishing pigs at 6.6 mg/kg dose was used to determine pharmacokinetic parameters in swine. A Cmax of 3.25 (±0.33) ug/mL was reported and AUC of 12.57 (±12.57) ug x h/mL was calculated in swine at the described dose. To

determine the concentrations a bioassay assay procedure was used with a limit of quantitation of 0.0125 µg/mL. When compared to sheep and cattle, the confidence interval for both the Cmax and the AUC was greater in swine, due to individual animal variation in swine (Martinez et al., 2001).

Apley et al. investigated ampicillin pharmacokinetics in swine by two injection methods (2007). A single intramuscular injection of ampicillin trihydrate at 17.6 mg/kg reconstituted to a concentration of 200 mg/mL was performed to compare to other routes of administration on the pharmacokinetics of ampicillin trihydrate in 21 day old pigs. HPLC was used for sample analysis with a limit of detection of 50 ppb and a limit of quantification of 100 ppb. Bioavailability along with pharmacokinetic parameters was determined from serial plasma sampling and resulting concentrations. The intramuscular bioavailability of ampicillin trihydrate was determined to be 83% (±29%). A half-life of 1.46 (±0.14) hours, an AUC of 25.07 (±3.09) h x ug/mL, a Cmax of 7.39 (±0.90) µg/mL, and a tmax of 1.0 (±0.5) hours were reported (Apley et al., 2007). When the dose of 6.6 mg/kg used in Martinez et al. was extrapolated to the parameters used in this study according to dose proportionality, a consistency of pharmacokinetic parameters was observed between the two studies (Apley et al., 2007).

A study was conducted in 2015 by Hamamoto and Mizuno to determine concentration of ampicillin in kidney, skeletal muscle, and intestine after oral administration at a 5 day withdrawal. Feeder pigs were administered oral ampicillin at 24 mg/kg/day for a week. Three pigs were euthanized five days following cessation of treatment and HPLC-MS/MS was used to determine tissue ampicillin residue concentrations. In muscle samples, 2 out of 3 pigs had detectable residues ranging from 0.09-1.8 ng/g, one pig had a positive residue in the kidney at 0.53 ng/g, and all three pigs had residues in the intestine which were all less than or equal to 1.93

ng/g. This research was conducted with the Japanese MRL's in mind, and determined that 5 days was sufficient withdrawal for oral administration of ampicillin to feeder pigs (Hamamoto and Mizuno, 2015).

A comparison of sodium ampicillin pharmacokinetic parameters and tissue residues by intramuscular or intravenous administration was performed by Galtier et al. (1979). Study animals were approximately 100 pound barrows. Four barrows were given a 20 mg/kg single intramuscular injection of sodium ampicillin, seven were given a 20 mg/kg single intravenous injection of sodium ampicillin, and four were given a 10 mg/kg single intravenous injection of sodium ampicillin. They determined that intramuscular injection resulted in lower rate constants than intravenous injection. They also described a two-compartment model of ampicillin metabolism when administered intramuscularly. In tissues of pigs receiving the 20 mg/kg intravenous injection, ampicillin residues were present at the highest concentration in kidney tissues. Tissues from the other two treatment groups were not sampled (Galtier et al., 1979).

Factors affecting drug metabolism

For all penicillins, absorption via the intramuscular route is much faster than the subcutaneous route, yet slower than intravenous injection. In some species it has been shown that penicillin absorption varies depending on the injection location as well. In horses injection of penicillin in the neck muscle results in a more rapid and complete absorption of the drug as compared to an injection in the rear leg or gluteal muscle (Firthe et al., 1986).

The route of drug administration may have profound effects on tissue residues and withdrawal times. For example, when injected subcutaneously, procaine penicillin G may cause local tissue reaction, inflammation, hematoma formation, or scar tissue formation. Any of these

reactions could result in a longer or incomplete absorption of the drug. Additionally, injection of a drug intermuscularly, such as in a fascial plane, can also result in delayed absorption due to poor blood supply to this area (KuKanich et al, 2005).

There may be several factors that influence drug absorption from the injection site. The first is the ability to absorb the injected volume of drug. There needs to be ample surface area to provide adequate absorption for the entirety of the injection volume to diffuse into surrounding tissues. The rate of movement through the tissues also affects drug absorption. If a high concentration of solute surrounds the unabsorbed injection drug, then the rate of absorption will decreased due to a decreased concentration gradient. This also presents the potential for osmotic pressure of the formulation affecting fluid volume at the injection site and subsequently absorption. An increase in fluid volume will decrease the rate of drug absorption. Likewise, a highly concentration injection will also cause delayed absorption due to decreased dissolution through surrounding tissues (Martinez et al., 2001).

With this in mind, absorption may differ based on species or age of animal just due to the practical application of needing more drug per unit weight. Larger animals may require a larger injection volume. Larger injection volumes could potentially affect the ability of the drug to be absorbed. Even though both procaine penicillin G and ampicillin trihydrate are administered as aqueous solutions, there is the potential to re-precipitate once they are injected in a subcutaneous or intramuscular location (Martinez et al., 2001).

Smith et al. (2014) examined the effect of different injection site patterns on penicillin absorption. The first injection site pattern was for the sows to receive an injection (up to 10 mL on either side of the neck) in the same location for each of the three consecutive days. The second pattern was also 10 mL on either side of the neck, but injections were placed 2 inches

apart on each consecutive day. The third injection pattern was up to 20 mL injection on one side of the neck with a 2-3 inch separation of injection on each consecutive day. There was no significant difference observed in percent positive KIS™ tests based on injection site pattern. However, there was some variability observed, which may be more due to the flip-flop kinetics of penicillin G absorption (Smith et al, 2014).

Another concern would be blood and lymph supply to the injected tissue. Good absorption depends on healthy circulation of blood and lymph. Exercise and differences in vascularization of different tissue types may affect this. Trauma to the tissue, increased vascular and lymphatic permeability, and inflammation of the injection site area may decrease absorption of the drug. Other tissue factors such as pH, temperature, and protein binding may affect drug absorption (Martinez et al., 2001).

Extrapolation of pharmacokinetic data from one species to another may not accurately reflect the drug absorption, metabolism, and elimination that are actually occurring. Some of the species differences include: different interaction with the drug product based on species-specific physiology, what makes up the rate-limiting step in each species, and an incorrect estimation of parameters due to differences in labeled doses for each species (Martinez et al., 2001).

Ampicillin pharmacokinetics and subsequently tissue residues may be affected by disease state. Previous studies of ampicillin residue depletion involved healthy animals. Any disease affecting the liver, kidney, heart, or circulatory system will affect the metabolism and distribution of the drug. Since ampicillin is eliminated through the kidney, renal disease or deficiency could greatly affect elimination (Yuan et al., 1997). Anemia, inflammation have been demonstrated to affect the drug in the plasma potentially altering drug distribution and protein binding. Endotoxin-related illness can also alter the pharmacokinetic properties of drugs. In

1997 Yuan et al. demonstrated that in pigs experimentally infected with *S. suum*, however, there was no difference in the pharmacokinetic parameters of ampicillin metabolism when administered by intramuscular injection compared to the uninfected group (Yuan et al., 1997).

Residue testing regulations

The Food Safety and Inspection Service (FSIS) provides guidelines for its National Residue Program (NRP) sampling guidelines. The NRP was created in accordance with the Federal Meat Inspection Act (FMIA), the Poultry Products Inspection Act (PPIA), and the Egg Products Inspection Act in order to protect consumer health and welfare. The NRP monitors and regulates all meat, poultry, and egg products that leave any establishments under federal inspection (Federal Register, 2012). The agencies that are in place to control the NRP are the FSIS, Food and Drug Administration (FDA), and Environmental Protection Agency (EPA). However, it is primarily the responsibility of the FSIS to enact and enforce the regulations described in the NRP to help control animal drug residues, environmental contaminants, pesticide, or any other chemical contaminants in food animal products (FSIS Directive 10,800.1). The residue sampling plan is reviewed annually, and a new plan is published each year. This annual sampling plan is created with historical annual residue information and information collected during FDA inspection on on-farm visits. The plan is published and is known as the Blue Book (Federal Register, 2012). The FSIS lists the veterinary drugs of interest for testing. Under the drug class of antibiotics, the beta-lactam group of pharmaceuticals is identified. Among the beta-lactams to be tested are both ampicillin and penicillin G (FSIS, 2015).

The Food and Drug Administration (FDA) has established accepted tolerances for veterinary drugs administered to animals entering the food chain. The tolerance is the level of

drug at which action may be taken against the supplier if a residue is detected. FSIS posts a list weekly of all suppliers that violate any tolerance levels (FSIS Notice 45-11). Maximum residue limits (MRL's) are values established for veterinary drug residues in animal tissues that are considered acceptable. MRL's are created with human safety in mind and are calculated based on what is considered and acceptable daily intake of each drug by humans and the maximum amount of animal product food intake a human may have (de Almeida et al., 2015).

Changes to the screening methods were first implemented by the FSIS with the switch to the Kidney Inhibition Swab (KIS™) test on September 11, 2011. At this time, all plants that slaughtered both cattle and swine and that were currently using the KIS™ test for screening of antibiotic residues in cattle would transition from the Fast Antimicrobial Screen Test FAST test to the KIS™ test for swine antibiotic screening (FSIS Notice 45-11). In July of 2012, the FSIS changed more of its testing methodologies for residue sampling. They began implementing multi-residue methods of analysis at FSIS laboratories. These methods are highly sensitive and selective and allow for the testing of a large number of compound types (FSIS Directive 10,800.1). They also replaced the FAST at the remaining plants with the KIS™ test (FSIS Directive 10,800.1).

There are three sampling program tiers to guide annual residue testing. The First Tier is the scheduled sampling program. The number of samples varies by animal class and drug class. Once this has been decided, an algorithm is used to determine the actual sampling times and locations. According to the Blue Book (2015), there are nine separate production classes to sample. One of these classes is sows. FSIS defines a sow as a "mature, female swine, ordinarily having given birth to one or more litters." In 2015, 800 sows were scheduled to be sampled and undergo routine residue testing using the MRM methods. Tier 2 is the targeted sampling plan,

and Tier 3 is the Targeted flock/herd. These last two tiers fall under the umbrella of inspector-generated sampling (FSIS, 2015).

Scheduled or directed sampling (Tier 1) is taken from animals that pass ante mortem inspection, and is a scheduled residue testing event. FSIS requires scheduled sampling and may target a specific slaughter class or chemical compound for which to sample, regardless of the post-mortem findings seen on carcasses. For these sampling procedures, inspection program personnel (IPP) are to collect a defined number and type of samples during a specified window of time (FSIS Directive 10,800.1).

Tier 2 and Tier 3 inspector-generated sampling are in place to allow for targeted sampling of carcasses that the inspector has reason to believe may contain a violative residue. This may include any animals that are from an owner that has previously had more than one confirmed residue violation in the past year, animals that are from a supplier on the Residue Repeat Violator list, or any animals with pathologies that may indicate reason belief of treatment. The following pathologies are listed by the FSIS that may warrant chemical residue testing: mastitis, metritis, peritonitis or surgery, injection site lesions, pneumonia, pleuritic, pericarditis, endocarditis, septicemia, generalized disease, injury or inflammation, cellulitis, beta-agonist use, or signs of treatment. Testing for residue should be conducting on any animals that appear to have signs of septicemia such as congested mucous membranes, altered body condition, depression and dehydration, hyperemia, or a low body condition associated with a systemic infection. For animals with injection site lesions, the IPP should not collect injection sites for residue testing. Rather, they should submit tissues from an area completely separate from the injection site (FSIS Directive 10,800.1).

Another type of sampling is the sampling of meat and poultry products that are imported to the United States. The guidelines for these are provided in a separate directive for the sampling of imported products. There is also a sampling strategy that involves sampling when national security is threatened. This is an emergency situation and is not regularly implemented (FSIS Directive 10,800.1).

In any case that an IPP suspects a residue violation, a public health veterinarian (PHV) or an IPP under the direction of a PHV is to perform an in-plant screening. The KIS™ test is used as a screening test for chemical residues. If an animal is selected for KIS™ testing, the plant must retain the carcass and parts until results are available. If the KIS™ test is positive the PHV or IPP should submit kidney, liver, and muscle for analysis at an approved FSIS laboratory. The FSIS laboratory will utilize multi-residue screening methods for the tissue samples. The carcass for any animal with a confirmed violative residue will be condemned. Any animal with a residue with no established tolerance will have the entire carcass and its parts condemned regardless of the location of the residue. No carcass or parts is allowed to enter the food chain unless testing at an FSIS laboratory confirms there are no violative residues in the tissue samples (FSIS Directive 10,800.1).

Guidelines for herd-level targeted residue testing are given in the FSIS directive. The number of animals to sample depends on group size. If there are 1-10 animals per group, only one animal is selected for KIS™ testing. Two animals are sampled in groups with 11-50 animals, 3 animals are sampled in groups of 51-100 animals, and four animals are sampled and subjected to KIS™ testing in any group larger than 100 animals (FSIS Directive 10,800.1).

Residue testing methodologies

There are two different types of tests that are used for drug residue testing. These include screening tests and confirmatory tests. An ideal screening test can detect a large variety of antimicrobials. It also needs to be quick and inexpensive to perform. The disadvantage of these screening methods are reduces sensitivity and specificity. The confirmatory methods have a much higher sensitivity and specificity and utilize liquid chromatography techniques in combination with mass spectrometry (de Almeida et al., 2015).

The Food Safety and Inspection Service (FSIS) uses liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a confirmatory test of penicillin G residues in animal tissues. It can be used to determine the level of residue present in bovine kidney, liver, and muscle tissues at levels as low as 25 ppb. A full description of the equipment and extraction process is detailed in CLG-PENG.1.01. The analytical range for penicillin G is described for kidney, liver, and muscle tissues at 15-400 ng/g, 30-1200 ng/g, and 5-200 ng/g, respectively (FSIS CLG-PENG 1.01).

There are many different described methods using LCMS for detection, which include a variety of validated tissue matrices for testing. For each testing methodology, the type of tissue used as a matrix must be validated. Good tissue matrices include muscle, kidney, liver, fat, and skin. This type of sampling method has two parameters, the limit of quantification (LOQ) and limit of detection (LOD), which describe the values at which the sample may be accurately and precisely quantified versus only analyte detection, respectively. One study examining ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) displayed very accurate and precise detection and quantification of both penicillin G and ampicillin residues in a swine kidney matrix (de Almeida et al., 2015).

There are multiple methods of detection and quantification of a wide variety of antibiotic residues in animal tissues. Many of the most sensitive and specific methods, including LC-MS/MS, are expensive and time consuming. In reality, very few of the animals selected for residue testing actually have a violative residue. For this reason, a good screening test it needed for antibiotic residue sampling (Schneider et al., 2009).

The KIS™ Test is a screening method originally designed to detect antibiotic residues in kidney tissue. It is the primary method of antibiotic screening used by the Food Safety and Inspection Service. It can detect residues in either kidney or muscle tissue of bovine, porcine, ovine, caprine, or poultry species, but is not extended for caprine muscle tissue (FSIS CLG-ADD 3.02).

The KIS™ Test uses bacterial inhibition as its method of detection. Either cooled or frozen tissues may be used. A swab is used to absorb kidney or muscle juice from the tissue. Each swab holds around 80 uL of sample. To swab the tissue, a ½ inch 1-2 cm deep circular cut is made in the tissue and the swab is placed in the cut area for around 30 seconds or until fully saturated. A bacterial culture in a liquid agar is prepared and the swab with juice sample is added to it, incubated at around 64°C, and then evaluated. For porcine kidney samples, the KIS test swab package indicates that an incubation time of 175 minutes is required (FSIS Directive 10,800.1). The pH change caused by bacterial growth causes the agar to turn yellow. If there are antimicrobials present in the tissues that cause an inhibition of bacterial growth, the agar stays a purple color. The kit comes with a reference card to compare the sample and determine color change. A negative result is a yellow agar, a positive sample is a purple agar, and a caution is when the bottom half of the agar is yellow and the upper half is purple. Any cautions should be interpreted as negative according to FSIS protocol (FSIS CLG-ADD 3.02). One observation by

Shelver et al. (2014) was a continued color change on KIS™ tests that were initially read as a "caution." Some caution tests darkened and were read as a positive test if read later in the 16 hour room-temperature grace period designated for reading the KIS™ test result (Shelver et al., 2014).

A positive result will require follow up according to established procedures and corrective action may take place. There is quite a range of sensitivities, as provided by the KIS™ Test manufacturer, depending on type of antibiotic. It is most sensitive against penicillin G residues, with the capacity to detect as little as 35 ppb in kidney tissue. Ampicillin can be detected at as low as 100 ppb in kidney tissue (FSIS CLG-ADD 3.02).

A study by Schneider et al. (2009) compares different screening methods in an experimental setting using bovine kidney juice and serum collected from slaughter plants. The two main tests of interest were the Fast Antimicrobial Screen Test (FAST) and the Kidney Inhibition Swab (KIS™) test. The FAST was used as the antimicrobial screening test of choice for the FSIS prior to the KIS™ test. FAST is performed by addition of the sample (kidney juice or serum) to a disc on an agar plate containing *B. megaterium* and then analyzing for zones of growth inhibition after 7 hours of incubation at 44°C. A test is considered positive with a zone of inhibition of greater than or equal to 10 mm around the sample disc. When compared to the KIS™ test, FAST was less sensitive on the confirmed positive residues than the KIS™ test for both kidney juice and serum samples. However, there were a number of false positive results on the KIS™ test for both sample types. It was also discovered that a positive sample that is incubated longer than the specified time may experience a color change to yellow, resulting in a false negative (Schneider et al., 2009).

Kidney juice, skeletal muscle juice, plasma, and urine from the 126 sows were subjected to the Charm KIS™ test for residue screening analysis by Smith et al. (2013). The KIS™ test sensitivity for penicillin in kidney juice is 20 ppb, 30 ppb in muscle juice, 20 ppb in urine, 30 ppb in serum, and 100 ppb in liver tissue. Historical data shows that penicillin residue concentrations in kidney tissues are typically 40-70 times higher than muscle tissues. The percentage of positive KIS™ test in kidney juice as compared to muscle juice would confirm this assumption as there were much fewer positive samples in the skeletal muscle tissue as compared to the kidney tissue. There were still KIS™ test positive samples on the last study date of 39 days post-treatment in kidney juice. Liver tissue returned the fewest KIS™ positive tests, concluding that it has much poorer sensitivity for penicillin G residue detection by KIS™ than the other tissue samples (Smith et al., 2013). LC-MS/MS was used to confirm positive samples. Through this it was determined that there were no false positive KIS™ tests in either urine or serum matrices. Urine served as a better representative sample for kidney juice than serum for penicillin G residue detection in sows with confirmed residues. There were a greater percentage of positive urine KIS™ tests than serum KIS™ tests at all time points. Because of this, serum was determined to be an ineffective ante mortem sample for penicillin G residue detection. This is likely due to a more rapid clearance of penicillin G from the serum than through the kidneys (Smith et al., 2013).

Alternative residue testing matrices

To determine the utility of frozen tissues for residue analysis, Shelver et al. (2013) investigated penicillin G residues in kidney tissues. Fresh kidney samples were compared to frozen kidney samples to evaluate the potential for delayed testing in frozen kidney samples.

Fresh kidneys were tested with KIS™ test at the time of sample collection following euthanasia. Frozen kidney samples were collected at the time of fresh kidney testing, but were frozen at -80°C until further analysis. Results determined that there was a consistent agreement of the positive KIS™ fresh kidney samples and the positive KIS™ frozen kidney samples, confirming that frozen kidneys may be used for residue analysis if fresh testing is not possible or in a research setting (Shelver et al., 2013).

The Schneider et al. study (2009) described serum as a potential matrix for ante mortem antibiotic residue testing. One trend that the investigators observed was a high variability of drug recovery and detection. Depending on hydration status, the volume of serum that could be removed from the sample varied with less volume available on more severely dehydrated animals. Animals with variable hydration statuses will result in a wide variability in serum volume, and thus increase the variability in serum as a sample type. However, their results showed that it has to potential to be a good matrix for ante mortem sampling, but animal hydration status and consistency of status must be taken into account (Schneider et al., 2009).

Musser et al. (2001) showed that penicillin remains in higher concentrations in the urine for a greater length of time than in either tissues or serum in a study of oral penicillin G administration in dairy calves. That study determined urine would make a good ante mortem sample for residue testing (Musser et al., 2001).

Oral fluids are a particularly useful sampling technique for several reasons. They reduce the need for individual animal restraint, which promotes human and animal safety. They are also useful for sampling populations of animals. Oral fluid samples have been repeatedly shown to provide diagnostic disease information about populations of animals. Oral fluid collection is implemented by hanging a cotton rope at the shoulder level of the pig(s) that are intended for

sampling. After 30-45 minutes, the rope is removed, wrung out in a plastic bag, and resulting fluid is collected in a small vial or container or analysis. An ideal volume of greater than 1 mL is desired (Pepin et al., 2014).

This idea is applied to breeding herds as well. One study by Pepin et al (2014) sampled 513 individually housed sows that had never been exposed to oral fluid collection previously. On the first day of collection, only 23.3% of sows had a successful collection and recovery of oral fluid sample. The same sows were sampled on the next day with a 47.8% success rate. This study also showed a predilection for younger sows to be more willing to chew on ropes than older sows. Further analysis is needed to optimize oral fluid collection in sows (Pepin et al., 2014).

Environmental sampling was designed to detect and identify pathogens in the environment. There are several methods of environmental sampling. One particular method has been described for detection of PRRSV in the environment. This sampling technique uses a commercial Swiffer® cloth to sample an environmental surface area. When compared to conventional environmental sampling with swabs, the Swiffer® cloth method proved to be more sensitive than the traditional swab. An advantage of the Swiffer® sampling is that it allows for a larger surface area to be sampled. It is also quite cost-effective and easy to perform (Kenney and Polson, 2011). To sample the environment with a Swiffer® cloth, the cloth is moistened with around 25 mL sterile saline or phosphate-buffered saline (PBS). A small area of the environment is selected and the wet cloth is used to scrub the selected area. The cloth is wrung out in a plastic bag, and the resulting fluid sample is collected in a tube or vial for further sample analysis, very similar to the oral fluid sampling technique (Kenney and Polson, 2011).

CHAPTER 2

PROCAINE PENICILLIN G RESIDUE DEPLETION IN TISSUES OF CULL SOWS WITH CORRELATION TO RESIDUE CONCENTRATIONS IN PLASMA, URINE, AND ENVIRONMENTAL SAMPLES

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Abstract

This project determined the tissue, plasma, and urine residue depletion of procaine penicillin G in cull sows at label and extra-label doses, with corresponding calculation of withdrawal times. We also examined the utility of urine, plasma, and environmental samples as matrices for residue analysis, the potential for environmental residue contamination, and the performance of rapid tests kits KIS™, Charm MRL™, and Snap™ test in detecting residues. Forty-seven sows were assigned to a treatment group receiving 3,000 IU/lb IM SID procaine penicillin G (n=15), 15,000 IU/lb IM SID procaine penicillin G (n=16), or a negative control group receiving sterile saline injections (n=16). Sows were sampled at 1, 6, 14, and 28 days post-treatment. With a 25 ppb action level, kidney withdrawal time for extra label dosing at 15,000 IU/lb was determined to be 30 days. Injection site withdrawal time was estimated to be 37 days. Urine and plasma could both serve as representative matrices for ante-mortem residue testing. Snap™ and Charm MRL™ test kits on urine returned similar results to LC-MS/MS testing, making them good ante mortem screening tests. Environmental samples are not a good tool for residue detection and there is no evidence to support the environmental transfer of penicillin G from treated to untreated sows.

Introduction

Procaine penicillin G is labelled for the treatment of erysipelas caused by *Erysipelothrix rhusiopathiae* (NADA 065-010). It is a broad spectrum antibiotic effective against gram positive and gram negative aerobes (Papich and Riviere, 2009). For this reason penicillin G has long been used extra-label for treatment of a wide range of swine pathogens, and is a common treatment used in sow herds. There is no established tolerance for penicillin G in the uncooked edible tissues of swine, so any detection of residue results in a violation. In 2011 the Food Safety and Inspection Service (FSIS) began changing its antibiotic residue screening test from the FAST to the Kidney Inhibition Swab (KIS™) test, which was fully implemented by July of 2012 (FSIS Notice 45-11). The change resulted in an increase in procaine penicillin G violative residues, particularly in cull sows at the time of slaughter, due to an increased sensitivity of residue detection. This prompted a need for more precise tissue residue analysis of penicillin G in swine tissues at extra-label regimens and extended withdrawal times. Smith et al. (2013) described tissue residues in sows at 39 days following administration of a 3 day course of penicillin G intramuscular injections at 5 times the labelled dose. They determined an extrapolated withdrawal time of 52 days with this dosing regimen would be sufficient for residue depletion below the 25 ppb action level (Smith et al., 2013).

The primary objective of this study was to determine the tissue residue depletion of procaine penicillin G for typical extra-label penicillin regimens in adult sows by directly measuring drug concentrations in kidney, liver, muscle (semimembranosus/semitendinosus), pelvic fat, and the injection site from the final day of administration with LC-MS/MS, and the development of withdrawal times from these data. Additionally, it is a goal to compare these drug concentrations derived from LC-MS/MS analysis to those results obtained by FSIS testing

methods, including the KIS™ test, Snap™ test, and Charm MRL™. This study will be able to compare the concentration of procaine penicillin G found in plasma, urine, and environmental samples to the tissue residue concentrations in an effort to determine the utility of these matrices for ante mortem residue detection and prevention. Finally, the study design employed in this study will allow for the evaluation of environmental transfer of procaine penicillin G to untreated sows when exposed to sows treated with common penicillin G treatment protocols.

Methods and materials

Before the initiation of this experiment, all animal use, handling, and sampling techniques described were approved by the Iowa State University Animal Care and Use Committee.

Animals and housing

Forty-seven (47) healthy cull sows were purchased from a commercial sow herd for enrollment in the study. None of the sows had previous antimicrobial treatment within a 52 day period prior to study enrollment. Sows were housed at the Livestock Infectious Disease Isolation Facility at Iowa State University. Sows were housed in four identical climate and humidity controlled rooms, each containing four pens of three sows. Each room corresponded to a sampling day and one sow from each treatment group was in each pen. They were allowed ad libitum access to water through a nipple drinker in each pen and were fed an age-appropriate non-medicated diet once daily that met or exceeded NRC nutrient requirements. Sows were weighed on arrival and were randomly allocated to treatment groups and sampling time points after blocking for weight. Blocking was performed to evenly distribute the effects of body size

on drug metabolism. Sows were tagged in the left ear with an individual identification number. Three one-inch diameter small circular tattoos were applied to the skin using a commercial slap tattoo applicator. Two were applied to the left and right post-auricular areas and one was applied to the right hip. A second tattoo of identical dimensions was pressed into the skin 4 inches ventral each of the first three tattoos to serve as an injection site for drug volumes that exceeded the specified limit.

Experimental design

Sows were randomized into one of three treatment groups. Within each treatment group, there were four groups corresponding to sampling time points 1, 6, 14, and 28 days following cessation of drug administration. Treatment Group 1 (n=11) received a 3,000 IU/lb IM dose of procaine penicillin G once daily for three days. Treatment Group 2 (n=12) served as the negative control group and received the sterile saline volume equivalent of a 9,000 IU/lb dose of procaine penicillin G IM once daily for three days. Treatment Group 3 (n=12) served as a representation of the dose that would be required to maintain appropriate antimicrobial drug concentrations above the MIC and received a 15,000 IU/lb IM dose of procaine penicillin G once daily for three days. One sow from Treatment Group 1, one sow from Treatment Group 2, and one sow from Treatment Group 3 were present in each pen to create an environment to test for potential environmental transfer of procaine penicillin G to the negative control sow resulting in a positive residue. Plasma and environmental samples were collected prior to drug administration, two days following final day of drug administration, and on each sow's sampling time point. Sows were necropsied on their scheduled sampling day for urine and tissue recovery.

Veterinary product

Procaine penicillin G (Agricillin, 300,000 IU/mL, AgriLabs, St. Joseph, MO, Lot #APG3M013, Expiration date: 12/2016) was the antibiotic product used for residue analysis. Procaine penicillin G is labeled in swine for treatment of erysipelas caused by *Erysipelothrix rhusiopathiae*. The labeled dose is 3,000 IU/lb for intramuscular injection once daily for up to four days. Sows were re-weighed within 24 hours prior to initiation of treatment to get accurate weights for dosage calculations. Each sow was restrained with a hog snare and individual injections were administered at a specified time for each individual. Injections were intramuscular and were given with an individually dosed syringe with a 16 gauge 1 inch needle inside a designated tattooed area to facilitate injection site recovery. Injections were administered for three consecutive days at the same time each day. The first day injections were administered in the left post-auricular area, the second day injections were administered in the right post-auricular area, and the third day injections were administered in the right hip area. For Treatment Group 1 up to 10 mL penicillin G was administered in the dorsal location and the remainder given in the ventral location. For Treatment Groups 2 and 3 up to 20 mL penicillin G was administered in the dorsal tattoo location and the remainder given in the ventral tattoo.

Sampling

Plasma. Blood samples were collected immediately prior to the first injection of penicillin, two days after completion of the dosing regimen, and immediately prior to euthanasia on each sow's designated sampling time point. Blood was obtained by left or right jugular venipuncture with a 16 gauge 4 inch needle and a 12 mL Luer lock syringe. Blood was immediately transferred to a 10 mL heparinized glass tube and inverted 5 times, labeled with a

unique identifier, and placed on ice. The blood was centrifuged at 1000g for 15 minutes, and the separated plasma was removed and placed in cryovials for storage at -80^{0}C until sample analysis could be performed.

Environmental Samples. Environmental sampling was performed in each group to assess the presence of procaine penicillin G in the environment after administration to sows. They were collected prior to drug administration, two days following cessation of penicillin G administration, and just prior to necropsy. An unscented Swiffer® pad was placed in a 50 mL vial containing 25 mL of physiologic saline until the pad was saturated with liquid. The wet pad was used to scrub a selected approximately area in the center of a pen with approximately 30 cm radius. Any excess fluid in the sampling area was mopped up by the pad and the pad was wrung out in a clean plastic bag to extract any absorbed fluid. The extracted fluid was poured off into a 50 mL plastic vial and stored at -80^0 C.

Urine. Urine samples were collected at or just prior to necropsy. An attempt was made to collect urine free-catch in a 50 mL plastic vial prior to euthanasia. If this was not obtained, pressure was applied to the urinary bladder over the flank 5-10 minutes following euthanasia to manually express the bladder, and a free catch sample was collected in a 50 mL plastic vial. Urine samples were stored at -80^{0}C until sample analysis.

Tissue Samples. Tissue samples were collected from sows at their assigned sampling time points 1, 6, 14, and 28 days following the final penicillin injection. Kidney, liver, skeletal muscle (semimembranosus/semitendinosus), pelvic fat, and the third injection sites (right hip) were sampled. A 2 inch circumference around the injection site tattoo and 3 inch deep section of tissue was dissected for injection site residue analysis. Both dorsal and ventral sites were

collected if injections occurred in both locations. Tissue samples were stored in Whirl-pak bags at -80^{0}C prior to analysis.

Drug extraction

Tissues. Calibration standards for tissue (liver, kidney, muscle, injection site) were prepared using standard additions of procaine penicillin G with 2 grams of ground/processed blank tissue. Blank tissue refers to tissue with no known exposure to penicillin G. Final concentrations of penicillin G were 1, 10, 50, 100, 250, 500, 1000 ng/mL. Standards were mixed using a vortex mixer and allowed to sit for 5 minutes. Internal standard, penicillin G –d7 ethylperidinium salt (Sigma, St. Louis, MO), was added to the standards/samples to give a final concentration of 500 ng/mL. Ten mL of acetonitrile:water (4:1) were added and standards/samples were mixed using a multi-tube vortexer for 5 minutes. Samples/standards were then centrifuged at 2500 rpm for 5 minutes. Supernatent was transferred to a 50 mL centrifuge tube containing 0.5 g of C18 sorbent. Ten mL of hexane saturated with acetonitrile was added. Samples/standards were vortexed for 1 minute and centrifuged at 3500 rpm for 5 minutes. Hexane was then aspirated to waste. Two mL of samples/standards were evaporated to dryness, resuspended in 50 µL of 25% (v/v) acetonitrile: water and 150 µL water and then transferred to an autosampler vial with glass insert. Samples were centrifuged for 20 minutes at 2400 rpm prior to LC-MS/MS analysis.

Plasma. Plasma samples were thawed and centrifuged. Aliquots of 500 µL were transferred to test tubes. Standards were prepared by adding penicillin G to 500 µL of blank plasma to obtain final concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng/mL. Quality control (QC) samples were prepared by adding penicillin G to 500 µL of blank plasma to obtain

final concentrations of 15, 150, 750 ng/mL. Internal standard, penicillin G-d7 ethylperidinium salt (Sigma), to give a final concentration of 40 ng/mL was added to each sample/standard. A volume of 2.5 mL acetonitrile was added to each standard/sample, followed by mixing with a vortex mixer, and then centrifugation for 20 min at 2400 rpm. Supernatant was transferred to a test tube and evaporated to dryness using a stream of nitrogen. Standards/samples were reconstituted in 50 µL of 25% (v/v) acetonitrile in water and mixed with a vortex mixer. Then 150 µL of water was added and mixed with a vortex mixer. Standards/samples were transferred to an autosampler vial with glass insert, centrifuged for 20 min at 2400 rpm and analyzed via LC-MS/MS.

Urine. Frozen standards/samples were thawed at room temperature. Standards for urine were prepared by spiking 150 µL of blank urine to concentrations of 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 10000 ng/mL penicillin G. QC samples were prepared at concentrations of 30, 300, 3000, and 30,000 ng/mL penicillin G by spiking 150 µL of urine. Internal standard, penicillin G-d7 ethylperidinium salt (Sigma), giving a final concentration of 250 ng/mL was added to each standard/QC/sample and then diluted 1:8 with water. Standards/QCs/samples were transferred to an autosampler vial with glass insert, centrifuged for 20 minutes at 2400 rpm and analyzed via LC-MS/MS. Samples that had a concentration higher than 10,000 ng/mL were diluted accordingly with water until their concentrations decreased enough to be in the range of the standard curve.

LC-MS/MS analysis

Concentrations of penicillin G in tissue samples were measured using LC-MS/MS. Separation was achieved via high-performance liquid chromatography (HPLC) using a Surveyor

pump and autosampler from Thermo Scientific (San Jose, CA, USA). Data collection was achieved using a Thermo TSQ Quantum Discover Max triple quadrupole mass spectrometer. The HPLC system utilized a Kinetex C18 column (100mm x 2.1 mm, 2.6 µm particle size) from Phenomenex (Torrance, CA, USA) maintained at 40 °C. The mobile phase consisted of A: 0.1% (v/v) formic acid in water and B: 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.25 mL/min. The mobile phase began at 20% B with a linear gradient to 95% B which was maintained for 2 minutes before re-equilibration to 20% B. Both penicillin G and penicillin G d-7, had a retention time of 4.6 minutes. The transitions used for penicillin G identification were (m/z) 335 → 114/160/176. The transitions used for the internal standard, penicillin G-d7, were 342 → 114/160/183. All data was collected in positive ion mode. All standard curves for tissues, plasma, and urine had a coefficient of determination that exceeded 0.98. QC samples were deemed to have passed when calculated concentration values were with 20% of expected levels.

Test kit analysis

KIS™. The Kidney Inhibition Swab (KIS™) test was performed on porcine kidneys, according to test instructions. The juice from porcine kidney, with no known exposure to antibiotics, was used as the negative control. Positive control was prepared by mixing 0.8 ml of juice from kidney with no known exposure to antibiotics with 0.5 ml of 50 ppb penicillin G. This solution of 50 ppb penicillin G, as well as other standards, with concentrations of 10, 20, 30 and 40 ppb were prepared in purified water by diluting a 1 ppt (part per thousand or 1 µg/µl) stock solution of penicillin G, potassium salt, in 60:40 acetonitrile:water. In each case, 0.5 ml of standard was mixed with 0.8 ml of kidney juice, not known to contain antibiotics. Following

incubation, the agar color was compared to the colors shown on the interpretation card included with the KIS™ test supplies. Results were interpreted as positive or negative.

Charm MRL™. Urine and environmental samples were analyzed according to test kit instructions. Standards were prepared by diluting a 1 ppt (part per thousand or 1 µg/µl) stock solution of Penicillin G, potassium salt, in 60:40 acetonitrile:water with water purified using a Millipore (Billerica,MA) Milli-Q water purification system. Standards had final concentrations of 1, 5, 10, 20, 30, 40, 50, 60, 100 and 200 ppb. Purified water was also used as the negative control. The positive control was prepared by dissolving one tablet of penicillin G, supplied in the test kit, in 1.0 ml of water purified using a Millipore (Billerica, MA) Milli-Q water purification system. 50 µl of sample were diluted with 450 µl of dilution buffer supplied in the test kit. 300 µl of this mixture was applied to the sample pad on the test strip. The strip was incubated for 8 min at 55°C, visually examined and then inserted into a ROSA™ Reader from Charm Sciences Inc. (Lawrence, MA). Prior to analysis, positive and negative calibration strips, supplied by the manufacturer, were read as a daily performance check. Samples were reported as positive or negative, according to the ROSA™ Reader result, as long as the positive control gave a reading greater than +400 and the negative control gave a reading less than -400, according to test kit instructions.

Snap™. Urine was centrifuged at 1200 g for 3 min prior to analysis. Environmental samples were mixed using a vortex mixer then centrifuged at 15000 rpm for 20 min prior to analysis. The New SNAP™ Beta-Lactam Test from IDEXX (Westbrook, ME) was used according to instructions. A disposable pipette provided with each SNAP™ device was used to draw up 450 µL (±50 µL) of sample. The sample was placed in the sample tube provided which contained a reagent pellet. After mixing thoroughly, the sample tube was incubated for 5 min at

45°C (±5°C). Following incubation, the sample was poured into the sample well on the SNAP™ device. As soon as the edge of the activation circle began to disappear, the activator was pushed down and the test device was left on the heater block for another 4 min. SNAP™ devices were verified visually and read using a SNAPshot™ Reader. Standards, with concentrations of 1, 5, 10, 20, 30, 40, 50 and 60 ppb were prepared in purified water by diluting a 1 ppt (part per thousand or 1 µg/µl) stock solution of penicillin G, potassium salt, in 60:40 acetonitrile:water. Purified water was used as negative control and to reconstitute the positive control, penicillin G, supplied in the test kit. Samples were reported as negative when the reading on the IDEXX SNAPshot™ Reader was 1.05 or lower, according to test kit instructions.

Statistical Analysis and Withdrawal Calculations

The data analysis for this paper was generated using SAS software, Version 9.4 of the SAS® System for Windows (Copyright © 2002-2014 SAS Institute Inc., Cary, NC, USA). Information was presented using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA). Withdrawal times were estimated for tissues by following the detailed method outlined in FDA Guidance for Industry #3 (FDA, 2006). For a time point to be included in a withdrawal calculation it should meet the following conditions: at least three sows must have quantifiable residues at each time point to include that time point in the linear regression, data points must follow a linear trend when plotted on a natural log scale, and at least three time points must be used to perform a linear regression. If less than three sows at one time point had residues above the LOQ, but a penicillin G residue below the LOQ and above the LOD was available from LC-MS/MS analysis, that value was used to perform withdrawal calculations in order to obtain a third time point. If a tissue residue was returned below the LOD, half the detection limit was

assigned for a numerical concentration for that tissue to obtain a third time point. These last two provisions were included in this analysis to meet the third time point requirement, although they do not strictly comply with the requirement for at least three animals to return residues above the limit of quantification. A small sample size reduced the ability to produce three sows with residues above the LOQ at three time points. According to FDA guidelines, withdrawal times were determined at the point when 99% of a population of treated animals would not test positive for a residue with a confidence of 95%. Withdrawal times were calculated for a depletion of residues to 25 ppb, 5 ppb, and 1 ppb, to correspond to the FSIS Minimum Level of Applicability (FSIS, 2013), the LOQ of the LC-MS/MS assay used in this trial, and the LOD of the LC-MS/MS assay used in this trial, respectively.

Results

Forty-seven sows were enrolled in the study. Due to this, only three sows were allocated to the 3,000 IU/lb treatment group for the day 1 sampling time point. The average sow starting weight was 238.7 kg ±4.0 kg. A summary of starting weights by treatment group and sampling time point are listed in Table 6.

Penicillin Residues by LC-MS/MS

Tissues. Kidney tissues in the treatment group receiving 3,000 IU/lb penicillin G, Treatment Group 1, tested positive for residues in at least one sow at each time point, out to 28 days post-treatment. The treatment group receiving 15,000 IU /lb penicillin G, Treatment Group 3, tested positive in at least one sow per sampling time point out to 14 days post-treatment. Liver penicillin G residues were only observed in samples (2/4 sows) from sows in Treatment group 3,

and were only observed on day 1. Penicillin G concentrations were only quantified in fat in Treatment Group 3 and were absent by day 14. Skeletal muscle penicillin G residues persisted to day 6 in the Treatment Group 1 sows and day 14 in the Treatment Group 3 sows. Injection site samples tested positive for penicillin G residues in both penicillin G treatment groups receiving at day 28. The penicillin G tissue residue depletion is displayed in Figure 1 and Figure 2 for Treatment Group 1 and Treatment Group 3, respectively. No sows in the negative control group, Treatment Group 2, had any penicillin G residues in kidney, liver, fat, or skeletal muscle at any time point. Four control sows had detectable penicillin G residues at injection sites, which were distributed from day 1 to day 28 post-treatment. Table 1 shows the LC-MS/MS concentrations in tissues by treatment group and sampling time point. The LOQ values established for kidney, liver, muscle, injection sites, and fat using this LC-MS/MS analysis method were 5 ng/g, 5ng/g, 5ng/g, 50 ng/g, and 5 ng/g, respectively.

Withdrawal time calculations were performed for kidney in Treatment Group 3 and skeletal muscle and injection sites for both treatment groups. Only the dorsal injection site penicillin residue concentrations were included since the maximum volume was administered in that location. Kidney and skeletal muscle tissues at day 14 for Treatment Group 1 and Treatment Group 3, and injection sites at day 14 for Treatment Group 1 did not meet all of the requirements needed for traditional withdrawal calculations. For these tissues values that were available but below the LOQ were still included in analysis. The withdrawal time calculated for kidney tissue was longer than skeletal muscle at all three minimum residue levels. For the 25 ppb cut-off, a withdrawal period of 30 days is needed for an extra-label 15,000 IU/lb IM SID dose for three consecutive days. In comparison if a cut-off of 1 ppb was used, kidney withdrawal time would be extended to 41 days. Injection site withdrawal times far exceeded any other tissue and ranged

from 37 to 44 days among all minimum residue concentrations and treatment groups. Table 2 lists calculated withdrawal times for each concentration.

Plasma. The plasma LOQ for this method was 5 ng/mL. Plasma penicillin G residues tested positive in 100% (4/4) of sows at day 6 for Treatment Group 1 and in 100% (4/4) sows at day 14 for Treatment Group 3. One sow in the negative control group tested positive for a low level of penicillin G in the plasma, 8.2 ng/mL, at day 6, but her previous plasma sample at day 3 tested negative for any residue. One control sow had plasma penicillin G concentrations of 24.5 ng/mL at time 0 and one Treatment Group 1 sow had a plasma concentration of 7.5 ng/mL at time 0. The control sow tested negative for penicillin G residues at day 3. Plasma penicillin G residues by time point are presented in Table 3.

Urine. The LOQ established for urine was 50 ng/mL. Penicillin G residues were found in 100% of samples at day 1 (4/4) and day 6 (3/3) for Treatment Group 1. All other time points tested negative. Penicillin G residues in Treatment Group 3 sows were detected out to Day 14. 100% of sows at day 1 (4/4) and day 6 (4/4) had very concentrated residues, ranging from a high of over 1 million ng/mL at day 1 to around 16,000 ng/mL on day 6. 50% (2/4) sows sampled on day 14 post-treatment had penicillin g residues present in the urine, and no sows had penicillin G residues present at day 28. The control group had no residues in urine samples in any sow at any time point. A complete list of urine residue concentrations is found in Table 4. A comparison of plasma and urine concentrations with tissue concentrations over time is displayed in Figures 3 and 4.

Environmental Samples. There were no detectable penicillin G residues detected in any of the environmental samples submitted for LC-MS/MS analysis.

Test Kit Residue Analysis

The Kidney Inhibition Swab (KIS™) test was run on frozen kidney tissue samples. For the treatment group receiving the labeled dose of penicillin, only kidney tissue from one sow (1/3) collected at one day following final injection penicillin G returned a positive result. When compared to LC-MS/MS residue levels, there were 5 positive kidney tissues that corresponded to a negative KIS™ test. When considering its sensitivity of only 35 ppb for penicillin G, only three of the kidney tissue residues were above that sensitivity level. In the extra-label dose group of sows, 6 sows had positive KIS™ tests. 100% of sows (4/4) at day 1 following final penicillin G injection were KIS™ test positive. 50% (2/4) of sows at day 6 following final penicillin G injection were positive, although one was a false positive and one was a false negative, although it corresponded to a LC-MS/MS residue level of 16.3 ng/g, which is below the assay sensitivity of 35 ppb. KIS test results are found in Table 5.

Charm MRL™ tests were performed on urine and oral fluids samples. Charm MRL™ tests on Treatment Group 1 urine samples were positive at day 6 (4/4 sows) but not at day 14 (0/4 sows). For Treatment Group 3, all sows at day 1 and day 6 tested positive on the Charm MRL™ test, and 75% of sows (3/4) tested positive at day 14. No sows tested positive at day 28. There was one false positive Charm MRL™ test in the control group, as confirmed by LC-MS/MS. All oral fluids samples tested negative for penicillin G residues on the Charm MRL™.

Urine and oral fluids were also evaluated for penicillin G residues by Snap™ tests. On urine samples, all sows at day 1 and day 6 for the 3,000 IU/lb treatment group tested positive for residues. 50% (2/4 sows) tested positive on day 14 and no sows tested positive by day 28. On the urine of the 15,000 IU/lb treatment group, all sows tested positive for penicillin G residues on

the Snap™ test at days 1, 6, and 14. 25% of sows (1/4) tested positive for residues at day 28, but not enough sample was left to confirm a positive by LC-MS/MS analysis. There were five false positive Snap™ tests in the negative control treatment group, which was confirmed with LC-MS/MS. A comparison of Snap™ and Charm MR™L test to LC-MS/MS results on urine samples are displayed in Figure 5 and Figure 6 For Treatment Group 1 and Treatment Group 3, respectively. Snap™ tests on oral fluids were negative at time 0, excepting two pens assigned to the sampling time point of day 14. These were considered false positives when compared to the negative test result of the LC-MS/MS analysis. On day 1, 33% (1/3) pens tested positive on the Snap™ test, 92% (11/12) pens tested positive on day 3, and 100% of pens (4/4) tested positive on days 6, 14, and 28. These were considered false positives since LC-MS/MS analysis found no penicillin G residues at any time points. Positive and negative predictive values were estimated for the test kits to determine the value of a positive or a negative test result. True positive or negative state was determined by the residue result returned from LC-MS/MS analysis. The PPV and NPV for the KIS™ test on kidney tissues was 0.86 and 0.83, respectively. For the Charm MRL™, the PPV and NVP were 0.89 and 1.0, respectively. The Snap™ test had a much lower positive predictive value than the Charm MRL™ at 0.62, but also was estimated to have an NPV of 1.0.

Discussion

Tissue residues

The primary study objective was to characterize the tissue residue profile of penicillin G at extra-label doses and to develop withdrawal times from these data. Penicillin G residues persists longest at the injection sites, which is reflected in the extended withdrawal times

calculated for these tissues. Residue depletion from the kidney was slightly longer than residue depletion from skeletal muscle. Penicillin G residues in fat and liver were quickly eliminated, but there were not enough sows that tested positive residues to calculate a numerical withdrawal time for residue depletion from these tissues. Penicillin G residues would not be expected in fat or liver since it has a low volume of distribution and elimination is primarily through the kidney.

A similar study by Smith et al. (2013) performed a penicillin G withdrawal study with the same dosing regimen. They calculated a withdrawal time of 52 days for kidney tissues after a 15,000 IU/lb IM injection of procaine penicillin G for three consecutive days using an established 25 ppb action level by the FSIS (FSIS, 2013). Using this same action level, the withdrawal time calculated for kidney tissues in this study with the same dosing regimen was 30 days. Discrepancy in the estimated withdrawal time could be a factor of sample size and study power.

Alternative sampling matrices

Plasma concentrations were consistently greater than tissue concentrations of penicillin G detected in kidney and skeletal muscle by LC-MS/MS at their respective time points. Residues were also detected in more sows at later time points in plasma than any of the tissue residues, excepting injection sites. With this information, plasma could function as an ante mortem sample for penicillin G residue detection. However, the only method used for plasma residue detection was LC-MS/MS analysis. Because a lab with specialized equipment and established residue analysis protocols is required for LC-MS/MS analysis, use of plasma as a practical, quick ante mortem sample may be limited. Comparison of the performance of plasma on the rapid test

kits would be an area of further investigation to determine the utility of this matrix for future penicillin G residue screening.

Urine and environmental samples were also assessed for use in ante mortem residue detection. Urine residues of penicillin G were found to correlate fairly well to plasma and tissue residues with LC-MS/MS analysis. Both rapid tests, Charm MRL™ and Snap™, had consistent results when detecting urine residues compared to the LCMS analysis. With this evidence, ante mortem urine testing with either of the rapid test kits could provide accurate information about penicillin G residues in the sow of interest. However, the Charm MRL™ had a much higher positive predictive value than the Snap™, which indicates that a positive on this test kit is more likely to actually reflect a an animal with a truly present residue than the Snap™ test with a much lower positive predictive value. Environmental samples were not an accurate representation of the tissue residues.

Test kits

Kidney Inhibition Swab (KIS™) tests on kidney tissue samples were less sensitive for residue detection than LC-MS/MS, but they were fairly good at screening for penicillin residues at higher tissue concentrations. The Charm MRL™ and Snap™ test on urine samples were very closely correlated to the results obtained with LC-MS/MS. They would be a viable option for ante mortem residue screening of urine, but Charm MR™L resulted in fewer false positives than the Snap™ test without a loss of sensitivity. Test kits did not perform well on the environmental samples. Since all Charm MRL™ tests of oral fluids were negative and a majority of Snap™ tests of oral fluids were positive, there is a disconnect between the two tests resulting in either a large number of false positive Snap™ tests, which would agree with LC-MS/MS results, or a

large number of false negative Charm MRL™ tests. Regardless, the results were not associated with any tissue residue or alternative matrix results and would not serve as a representative sample for penicillin G residue testing. The high number of suspected false positives observed primarily with the Snap™ test could be due to a cross reaction of another substance in the samples which resulted in a positive reading. Further investigation is needed to elucidate the false positive findings.

Environmental contamination

Treatment Group 2, the negative control group, was included in the study design to examine the potential for environmental contamination by penicillin G after injection in a neighboring sow, and its subsequent ability to cause residues in non-treated sows. Anecdotally some violative residues at slaughter have been attributed to a theory that this has occurred in non-treated sows. Negative control sows were housed in a pen with one sow treated with the labeled dose of penicillin G and one sow with the extra-label dose of penicillin G in order to create an environment that would have a likelihood of penicillin G contamination. Environmental contamination was evaluated with environmental sample collection and penicillin G residues were evaluated by tissue, plasma, and urine LC-MS/MS analysis for penicillin G.

The plausibility that a treated sow could lead to an environmental transfer of penicillin G to an untreated sow and cause residues in that sow is very low. All environmental samples were negative for penicillin G residues after LC-MS/MS analysis. This method of residue testing is highly sensitive and specific and was considered the reference standard for residue levels. With all samples negative, the likelihood that penicillin G could survive metabolism by the treated sow and persist in the environment is extremely low. The environmental sample matrix has not

been well-studied, and there is a potential that penicillin G stability and extraction from this matrix affected the ability to detect any residues in the environment by LC-MS/MS. Penicillin G can also be unstable over time, and although the samples were stored at -80°C, there may have been breakdown of the molecule which caused the negative test results for penicillin G residues in the environmental samples.

Further evidence to disprove the notion penicillin G residues result from environmental transfer, there was no evidence of tissue residues in kidney, liver, skeletal muscle, fat, or urine in any of the control sows at any time points. The one positive plasma sample in a control sow prior to any penicillin G injection could not have been a result of environmental contamination since no sows would have received any penicillin at this time. The one slight positive plasma sample at day 6 is most likely a false positive or a result of a laboratory error since the same sow's plasma sample tested negative on day 3. Four negative control sows tested positive for injection site residues. Hip injection sites were specifically collected for tissue residue analysis to rule out any residues from prior treatments since any unknown treatment prior to study enrollment would have occurred in the neck and not in the hip. The positive results that were seen could have been due to residual penicillin G residues in the separation column used for analysis. The laboratory was blinded to treatment group and samples were processed according to a randomly assigned numerical order. Some of the treated sows had very concentrated injection site residues and processing a negative control sow just after a highly concentrated sow may have resulted in a false positive due to contamination.

Acknowledgments

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CHAPTER 3

TISSUE RESIDUE DEPLETION PROFILE OF AMPICILLIN TRIHYDRATE IN CULL SOWS AND COMPARISON WITH PLASMA, URINE, AND ORAL FLUID RESIDUES

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Abstract

The tissue residue depletion profile of ampicillin trihydrate in cull sows and the utility of other sampling matrices plasma, oral fluids, and urine samples was determined. A KIS™ test, Charm MRL, and Snap test kits were compared with results obtained by LC-MS/MS. Sixty cull sows in two treatment groups received a 6 mg/kg (n=30) dose of ampicillin trihydrate and 12 mg/kg (n=30) dose of ampicillin trihydrate once daily for three days. Three sows from each treatment group were sampled at 1, 3, 5, 8, 10, 12, 15, 20, 30, and 40 days following the final day of treatment. Ampicillin residues were not detected in kidneys after day 1 following treatment. Injection site residues persisted at 40 days. Withdrawal times were estimated for injection sites at 41 and 89 days for a 6 mg/kg and a 12 mg/kg dose, respectively. Plasma ampicillin residue concentrations were similar to kidney ampicillin residues. KIS™ tests on kidney samples did not detect ampicillin residues at concentrations of 142 ng/g or lower. Charm MRL™ tests on urine were more accurate at detecting urine ampicillin concentrations than the Snap™ tests, and Snap™ tests returned a high number of false positives. Ampicillin residues were not detected in oral fluids samples.

Introduction

With an increase in penicillin G residue violations at slaughter, ampicillin trihydrate is a commonly utilized alternative for antibiotic treatment of sows. There is no approved ampicillin trihydrate product currently available for use in swine, so all use must comply with AMDUCA regulations on extra-label drug use. Ampicillin is an aminopenicillin used in swine for the treatment of gram positive and gram negative aerobic bacterial infections with significant efficacy against *Pasteurella multocida*, beta hemolytic *Streptococcus* spp., and *Streptococcus suis*. Ampicillin has moderate efficacy against *Salmonella typhimurium*, and contrary to previous MIC data, has low efficacy against *E. coli*, *Salmonella cholerasuis*, or *Actinobacillus pleuropneumonia* (Salmon et al., 1995). Annual sensitivity data reported by the Iowa State University Veterinary Diagnostic laboratory showed most bacterial isolates tested to be highly sensitive to ampicillin and in all isolates tested there was a greater sensitivity to ampicillin than penicillin G (Iowa State University, 2014).

Despite its effectiveness against swine pathogens, very little pharmacokinetic or tissue residue data is available for intramuscular injection of ampicillin trihydrate in swine. Martinez et al. (2001) provided pharmacokinetic parameters after a singular intramuscular injection of 6.6 mg/kg dose of ampicillin trihydrate (100 mg/mL aqueous suspension) to six mature pigs. Apley et al. (2007) investigated a single intramuscular injection of a 17.6 mg/kg dose of ampicillin trihydrate to 21 day old pigs and derived similar pharmacokinetic parameters. However, neither study was performed in sows or over an injection course of multiple days, which is usually required to maintain adequate drug concentration for antimicrobial efficacy. There is also no information on ampicillin tissue residue depletion following intramuscular administration in

swine. Current FARAD recommendations for ampicillin meat withdrawal times after a 6 mg/kg IM dose and a 12 mg/kg IM dose SID for three days are 16 days and 21 days, respectively.

The objectives of this study are to describe the tissue depletion profile of ampicillin in sows following intramuscular administration of ampicillin trihydrate at 6 mg/kg and 12 mg/kg once daily for three consecutive days and to correlate the tissue residue concentrations with plasma, oral fluids, and urine ampicillin residue concentrations using LC-MS/MS over 40 days following drug administration. It is also the goal to determine the utility of using the antimicrobial screening tests KIS™, Charm MRL™, and Snap™ to provide reliable tissue, oral fluids, and urine residue results when compared to LC-MS/MS.

Methods and materials

Before the initiation of this experiment, all animal use, handling, and sampling techniques described were approved by the Iowa State University Animal Care and Use Committee (IACUC # 3-14-7757-S).

Veterinary product

Ampicillin trihydrate (Polyflex, Boehringer Ingelheim Vetmedica, Lot #p5181, Expiration date: 12/1/2017) was the antimicrobial drug for residue analysis. Ampicillin trihydrate does not have a label for use in swine, but is approved for the treatment of respiratory tract infections and bacterial pneumonias susceptible to ampicillin in cattle at a dose of 2-5 mg/lb IM SID for up to seven days. Prior to drug administration sows were re-weighed for accurate dosage calculations. Ampicillin injections were given once daily for three days at the same time each day. The ampicillin product was reconstituted to a concentration of 200 mg/mL.

Intramuscular injections were given via individually dosed syringes with a 16 gauge, 1 inch needle inside the circular tattoo that was placed on arrival to ensure accurate recovery of the injection site. All injections up to 10 mL were administered on the left post-auricular area and remaining volume of drug for injection was administered on the right post-auricular area. Sows were restrained with a hog snare for drug administration and sample collection.

Animals and housing

Sixty (60) healthy cull sows were obtained from a commercial sow, and had no previous known antimicrobial treatment within 52 days prior to enrollment in the study. Sows were placed in study pens upon arrival and were acclimatized for 72 hours. Each sow was identified by the use of a plastic livestock ear tag placed in the left ear of the sow at arrival (Allflex small round button tag; Allflex Dallas, TX). A one-inch diameter circular tattoo was placed on the post-auricular area on both sides of the neck in the skin above the trapezius muscle using a commercial tattoo applicator to indicate drug injection site. Sows were housed in groups of three (3), according to assigned group, in a humidity and climate-controlled environment at the Livestock Infectious Disease Isolation Facility at Iowa State University. Housing conditions and live animal care and procedures were in accordance with the protocol approved by the Iowa State University Animal Care and Use Committee (IACUC # 3-14-7757-S). Sows were fed an age-appropriate non-medicated diet once daily that met or exceeded NRC nutrient requirements. Sows had ad libitum access to water through a nipple waterer in each pen. Upon arrival sows were weighed to the nearest 0.1 pound. Sows were randomly assigned to a treatment group and sampling time point after blocking for weight. Blocking was performed to reduce effects of body mass and parity on drug metabolism.

Experimental design

Sows were allocated to one of two treatment groups. Treatment Group 1 (n=30) received a 6 mg/kg dose of ampicillin trihydrate (200 mg/mL suspension) and Treatment Group 2 (n=30) received a 12 mg/kg dose. Within each treatment group, three (3) sows were allocated to one of ten (10) sampling groups corresponding to 1, 3, 5, 8, 10, 12, 15, 20, 30, and 40 days following the final day of drug administration. Plasma and oral fluids samples were collected prior to drug administration and on each sow's sampling time point. Sows were necropsied on their scheduled sampling day for urine and tissue recovery.

Sampling

Blood samples were obtained immediately prior to the first administration of ampicillin and immediately prior to euthanasia. 8 mL of blood was collected from the left or right jugular vein using a 4 inch 16 gauge hypodermic needle and a 12 mL Luer lock syringe. Blood samples were collected in glass 10 mL heparin tubes. The samples were mixed by inverting the tube, labeled with a unique identifier, and immediately placed on ice. The blood samples were centrifuged and 1000g for 15 minutes, and the separated plasma was stored in cryovials at -80^{0}C until sample analysis.

Oral fluids were collected for residue analysis in each group of three sows to assess for drug concentrations in this matrix. Oral fluid samples were collected just prior to administration of ampicillin and just prior to the final blood sampling time point before necropsy. Sows were allowed to chew on cotton fabric, and fluids were extracted by wringing out the fabric in a clean plastic bag and pouring the resulting fluids into a 50 mL Falcon tube. A minimum of 5 mL of fluid was considered acceptable. Oral fluids were stored at -80^{0}C until sample analysis.

Urine samples were collected at or just prior to necropsy. An attempt was made to collect urine free-catch in a 50 mL Falcon tube prior to euthanasia. If this was not obtained, pressure was applied to the urinary bladder over the flank following euthanasia and a free catch sample was collected in a 50 mL Falcon tube. If no urine was obtained through either of these methods, urine was aspirated from the urinary bladder with a 6 mL Luer lock syringe with a 22 gauge ¾ inch needle. Urine samples were transferred to a non-additive red top tube and stored at -80^{0}C until sample analysis.

Tissue collection occurred at the designated necropsy and sampling time points corresponding to 1, 3, 5, 8, 10, 12, 15, 20, 30, and 40 days after final drug administration. Sows were euthanized by captive bolt and exsanguination. Kidney, liver, muscle (semimembranosus/semitendinosus), and the injection site(s) were collected and submitted for analysis. A two-inch circumference, 5 inch deep section of tissue around the injection site tattoo was dissected out for sampling. These tissue samples were stored Whirl-Pak bags and placed on ice until permanent storage at -80^{0}C for sample analysis.

Tissue analysis

Calibration standards for tissue (liver, kidney, muscle, injection site) were prepared using standard addition of ampicillin with 2 grams of ground/processed blank tissue. Blank tissue refers to tissue with no known exposure to the ampicillin. Final concentrations of ampicillin were 5, 10, 50, 100, 250, 500, 1000 ng/mL. Standards were mixed using a vortex mixer and allowed to sit for 5 minutes. Internal standard, penicillin V, was added to the standards/samples at a final concentration of 500 ng/mL. Two mL of water were added to standards/samples and mixed using a vortex mixer. Eight mL of acetonitrile were added and standards/samples and

mixed using a multi-tube vortexer for 5 minutes. To help remove excess fat on muscle and injection site tissues, 10 mL of hexane saturated with acetonitrile, was mixed with samples/standards. The hexane was removed by aspiration. Injection site samples that exceeded the highest standard were re-extracted using less tissue (0.5 g) and/or diluted with blank tissue extract containing internal standard. All samples/standards were centrifuged at 2500 rpm for 10 minutes and then passed through a preconditioned solid phase extraction (SPE) cartridge containing polymeric reversed phase material (Strata-X 8B-S100-FBJ). Samples/standards were evaporated to dryness, resuspended in 200 µL of 12.5% (v/v) acetonitrile: water and refrigerated for 20 minutes. After refrigeration, samples were centrifuged for 20 minutes at 2400 rpm and transferred to an autosampler vial with glass insert for analysis on LC-MS/MS.

Concentrations of ampicillin in tissue samples were measured using LC-MS/MS. Separation was achieved via high-performance liquid chromatography (HPLC) using a Surveyor pump and autosampler from Thermo Scientific (San Jose, CA, USA). Data collection was achieved using a Thermo TSQ Quantum Discover Max triple quadrupole mass spectrometer. The HPLC system utilized a Kinetex C18 column (150mm x 2.1 mm, 2.6 µm particle size) from Phenomenex (Torrance, CA, USA) for liver, kidney, and muscle samples, while a shorter column (100mm x 2.1 mm, 2.6 µm particle size) was used for injection site samples. Both columns were maintained at 40 °C. The mobile phase consisted of A: 0.1% (v/v) formic acid in water and B: 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.25 mL/min. The mobile phase began at 5% B with a linear gradient to 50% B which was maintained for 1 minute and then increased linearly to 95% B. The flow rate was increased to 0.3 mL/min and re-equilibration to 5% B was allowed to take place. For ampicillin and penicillin V, retention times were 5.95 and 9.20 minutes, respectively on the 150 mm column and 4.70 and 8.50 minutes, respectively on the 100

mm column. The transitions used for ampicillin identification were (m/z) 350 → 79/106/114/. The transitions used for the internal standard, penicillin V, were 351 → 87/114/160. All data was collected in negative ion mode. Standard curves ranged from 5-1000 ng/mL for ampicillin in tissue.

Plasma, oral fluids, and urine analysis

Plasma samples were thawed and mixed using a vortex mixer. Aliquots of 150 µL were transferred to a vial and 500 µL of 500 ng/mL internal standard, penicillin V in acetonitrile with 0.1% (v) formic acid was added to each sample. Standards were prepared by adding ampicillin to 150 µL of blank plasma to obtain final concentrations of 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL. Quality control (QC) samples were prepared by adding ampicillin to 150 µL of blank plasma to obtain final concentrations of 30, 300, and 1500 ng/mL. Standards/samples were mixed with a vortex mixer and centrifuged at 2400 rpm for 20 minutes. Supernatant was transferred to a test tube and evaporated to dryness using a stream of nitrogen. Standards/samples were reconstituted in 75 µL of 25% (v/v) acetonitrile in water and mixed with a vortex mixer. Then 75 µL of water was added and mixed with a vortex mixer. Standards/samples were transferred to an autosampler vial with glass insert, centrifuged for 20 min at 2400 rpm and analyzed via LC-MS/MS.

The extraction method for urine and oral fluids used a strong cation exchange cartridge which does not retain penicillin V. Therefore, cephalexin was used as the internal standard for urine and oral fluids. Frozen standards/samples were thawed at room temperature and mixed vigorously. Standards for urine were prepared by bringing 1 mL of urine to final concentrations of 5, 10, 20, 50, 100, 200 ng/mL ampicillin. Higher concentrations were outside the linear range.

QC samples were prepared by bringing 1 mL of urine to final concentrations of 15 and 150 ng/mL ampicillin. The 1 mL standards, QCs, and samples were spiked with 250 ng of internal standard, cephalexin, and diluted with 1.5 mL of acetic acid. SPE was performed using Strata-X-C 33u polymeric strong cation exchange columns (100 mg/3 mL) from Phenomenex (Torance, CA, USA) were preconditioned with 1 mL of methanol and equilibrated with 1 .5 mL 1 M acetic acid. Standards/samples were loaded using gravity flow. The standards/samples were washed with 1 mL 1 M acetic acid, 1 ml 70% (v/v) acetonitrile in water, and 1 mL methanol. The SPE cartridges were then dried under nitrogen for 5 minutes. Standards/samples were eluted using two aliquots of 0.75 mL 4% (v/v) ammonium hydroxide in 70% (v/v) acetonitrile in water, evaporated to dryness. Standards/samples were reconstituted in 75 µL 25% (v/v) acetonitrile, mixed with a vortex mixer. Then 75 µL of water was added and mixed with a vortex mixer. Standards/samples were transferred to an autosampler vial with glass insert, centrifuged for 20 minutes at 2400 rpm and analyzed via LC-MS/MS.

Concentrations of ampicillin in plasma, urine, and oral fluids were measured via LC-MS/MS, utilizing a Finnigan LTQ ion trap mass spectrometer from Thermo Scientific (San Jose, CA, USA), coupled to an Agilent 1100 series pump and autosampler (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of A: 0.1% (v/v) formic acid in water and B: 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.25 mL/min. The mobile phase began at 7.5% B with a linear gradient to 95% B at 8.75 minutes, which was maintained for 2 minutes at an increased flow rate of 0.325 mL/min, followed by re-equilibration to 7.5% B. Separation was achieved with an ACE3 C18 column (150 mm x 2.1 mm, 3 µm particle size) from Advance Chromatography Technologies, LTD (Chadds Ford, PA, USA) maintained at 40°C.

For ampicillin, cephalexin, and penicillin V, retention times were 5.50, 5.49, and 8.37 minutes, respectively. The following transitions were used for identification: ampicillin (m/z) 350 →106/160/192, cephalexin (m/z) 348 → 148/158/190 and penicillin V (m/z) 349 →208/305. Ampicillin and cephalexin data collection was in positive ion mode. Penicillin V data collection was in negative ion mode. Standard curves ranged from 5-5000 ng/mL for plasma and 5 -200 ng/mL for urine and oral fluids. All standard curves for tissues, plasma, urine, and oral fluids had a coefficient of determination that exceeded 0.98. QC samples were deemed to have passed when calculated concentration values were with 20% of expected levels.

Test kit analysis

KIS™. The Kidney Inhibition Swab (KIS) test was performed on ground porcine kidney samples, according to instructions. Porcine kidney with no known exposure to antibiotics was used as the negative control. Ground negative control kidney, with 50 ppb penicillin G added and mixed, was used as the positive control. Following incubation, the agar color was compared to the colors shown on the interpretation card included with the KIS test supplies. Results were interpreted as positive or negative.

Charm MRL™. Urine standards were prepared in blank adult porcine urine obtained from Lampire Biological Laboratories, Inc. (Pipersville, PA). This urine was also used as the negative control. For oral fluid samples, oral fluids were centrifuged at 3400 rpm for 53 min and filtered prior to analysis, using 0.2 µm Mini-UniPrep syringeless nylon filters with polypropylene housing from Whatman (Florham Park, NJ). Blank oral fluids obtained from the Swine Medicine Education Center (SMEC) at Iowa State University were used as the negative control, as well as to prepare standards. The positive control was prepared by dissolving one

tablet of penicillin G, supplied in the test kit, in 1.0 ml of water purified using a Millipore (Billerica, MA) Milli-Q water purification system. 50 µl of sample were diluted with 450 µl of dilution buffer supplied in the test kit. 300 µl of this mixture was applied to the sample pad on the test strip. The strip was incubated for 8 min at 55°C, visually examined and then inserted into a ROSA™ Reader from Charm Sciences Inc. (Lawrence, MA).

Snap™. Urine was centrifuged at 3400 rpm for 20 min prior to analysis. Oral fluid samples were centrifuged at 2770 rpm for 52 min and filtered prior to analysis, using 0.45 µm, 17 mm nylon syringe filters from Thermo Scientific (Rockwood, TN). The New SNAP™ Beta-Lactam Test from IDEXX (Westbrook, ME) was used according to instructions. A disposable pipette provided with each SNAP™ device was used to draw up 450 µL (±50 µL) of sample. The sample was placed in the sample tube provided which contained a reagent pellet. After mixing thoroughly using a vortex mixer, the sample tube was incubated for 5 min at 45°C (±5°C). Following incubation, the sample was poured into the sample well on the SNAP™ device. As soon as the edge of the activation circle began to disappear, the activator was pushed down and the test device was left on the heater block for another 4 min. SNAP™ devices were verified visually and read using a SNAPshot™ Reader. Two control matrices were used as negative controls and to reconstitute the positive control, penicillin G, supplied in the test kit. One was antibiotic-free whole milk purchased at a local store. The other was water that had been purified using a Millipore (Billerica, MA) Milli-Q water purification system.

Statistical Analysis

The data analysis for this paper was generated using SAS software, Version 9.4 of the SAS® System for Windows (Copyright © 2002-2014 SAS Institute Inc., Cary, NC, USA). Data is presented using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA). Withdrawal periods were estimated using the method described in the FDA Guidance for Industry describing the process for estimation of a withdrawal period (FDA, 2006).

Results

Sows assigned to sampling time points 1, 3, 5, 8, and 10 days post-treatment were sourced from an older parity commercial sow source. Due to housing restrictions, sows assigned to sampling time points 12, 15, 20, 30, and 40 were analyzed during a separate time period and were sourced from a younger parity commercial sow source. There is no significant difference in starting sow weights between the two treatment groups, but a significant difference was observed in starting weights from the first five sampling time points to the second five sampling time points, as shown in Table 12.

Tissue analysis

Analysis of tissues by LC-MS/MS was able to quantify ampicillin residues in tissues at a level of quantification of 5 ng/g tissue. Ampicillin residues were detected in kidney tissues of both treatment groups (6/6 sows) at one day following the final ampicillin treatment. No other kidney samples at any other time points tested above the LOQ for ampicillin. Figure 7 displays the ampicillin residue depletion over forty days in the kidney. Injection sites samples tested positive for ampicillin in at least one sow from each treatment group at all time points out to 40

days following the final injection of drug. In the 6 mg/kg treatment group, an ampicillin residue level of 52 ng/g was detected in 33% (1/3) of sows. In the 12 mg/kg treatment group, an ampicillin residue level of 51 mg/g was detected in 33% (1/3) of sows. Injection site ampicillin residue depletion is shown in Figure 8. No ampicillin residues above the LOQ were found in either liver tissue samples or skeletal muscle tissue samples. Ampicillin tissue residue concentrations by sow and tissue type are displayed in Table 7. Injection site tissues were the only sample type to meet the requirements for withdrawal time calculation and analysis. Withdrawal times were determined to be the value when 99% of the population of animals would no longer test positive for a drug residue above the ampicillin tolerance limit with a 95% confidence. In sows with two injection sites, only values from the injection site receiving the maximum volume of injection (10 mL) was included for calculations. Values from days 1, 3, 5, 8, 10, and 12 were used to determine a minimum of 41 days for ampicillin residues to deplete below 10 ng/g tissue in injection sites given the 6 mg/kg dose. Values from days 1, 3, 5, 8, 10, 12, and 15 were included in the calculation to determine that 89 days are needed following final injection of ampicillin at a dose of 12 mg/kg for ampicillin residues to deplete below 10 ng/g tissue. To meet inclusion requirements for withdrawal calculations, all three sows at each time point had to have ampicillin residues above the LOQ and they had to follow a linear pattern when plotted on a natural log scale. Withdrawal time results are found in Table 8.

Plasma, oral fluids, and urine analysis

In both treatment groups, 100% (6/6) of sows tested positive for ampicillin residues above the LOQ at day 1 following drug administration. One sow (1/3) had an ampicillin residue level of 18 ng/mL in plasma at day 3 following the final injection of drug. There were no other

positive plasma samples at any other time points in either treatment group. A complete list of plasma concentrations is found in Table 9. Urine samples had detectable residues out to ten days following final injection of ampicillin. In the 6 mg/kg treatment group a residue level of 8 ng/mL was quantified in 1/3 sows and in the 12 mg/kg treatment group a residue level of 10 ng/mL was quantified in 1/3 sows. Urine residue concentrations are listed in Table 10. No oral fluids samples had detectable ampicillin residues at any time point.

Test kit analysis

The KIS™ test on kidney tissue returned a result of "positive" in one sow (1/3) in the 6 mg/kg treatment group at day 1 post-treatment which correlated with an LC-MS/MS determined residue concentration of 945 ng/g ampicillin. An "uncertain" result was returned in another sow of the same treatment group and sampling time point. All other KIS™ tests were negative. There was a 16.7% agreement between LC-MS/MS positive samples and KIS™ test positive samples, with 5 false negative KIS™ tests. The Table 11 displays the KIS™ test results. Charm MRL™ tests on urine samples returned positives out to day 8 of the 6 mg/kg treatment group (3/3 sows) and out to day 12 of the 12 mg/kg treatment group (1/3 sows). The Snap™ tests on urine samples were positive at day 30 in both treatment groups. Figure 9 and Figure 10 shows the percent positive Charm MRL™ and Snap™ tests compared to percent positive urine samples on LC-MS/MS for Treatment Group 1 and Treatment Group 2, respectively. Positive predictive value (PPV) was calculated do determine the likelihood of a positive test representing an animal that truly has a tissue or urine residue result, and negative predictive value (NPV) was calculated to determine the likelihood of a negative test representing an animal that truly has a negative residue result, using LC-MS/MS as the reference for true residue status. The KIS™ test

had a PPV of 1.0 and an NPV of 0.92. Snap and Charm MRL PPV and NPV were estimated using urine residue results. The PPV and NPV for the Charm MRL were 0.88 and 0.94, respectively. The PPV and NPV for the Snap were 0.46 and 1.0, respectively.

Discussion

Tissue residues

Ampicillin trihydrate appears to be rapidly metabolized and eliminated from muscle tissues and kidneys. This is displayed with no detectable residues in skeletal at any time point in either treatment group, or residues detected in kidneys only at one day following completion of the ampicillin treatment regimen at both treatment doses. Ampicillin elimination occurs primarily through the kidneys, so little to no residues were expected in the liver, which was seen with the LC-MS/MS analysis. Injection site residues were present in at least one sow from every treatment group and were quantifiable at least 40 days following final drug administration. There was also a wide variability among injection site residue concentrations within sows at the same time point. The trihydrate molecule used in the ampicillin formulation for injection in this study is included to increase stability and slow absorption of ampicillin from the injection site, prolonging the rate at which the drug enters the plasma (Papich and Riviere, 2009). By extending absorption, ampicillin can remain in plasma at concentrations above the MIC for bacteria for which the drug is indicated. However, the trihydrate formulation could contribute to the extended detection of drug at the injection site. There are a variety of factors that could extend ampicillin residues at the site of injection. Volume of injection, highly concentrated injectable solutions, re-precipitation of the ampicillin trihydrate product, and local tissue reaction could directly affect absorption rate (Martinez et al, 2001). Accidental intermuscular or

subcutaneous injection of ampicillin trihydrate, rather than intramuscular, could also result in increased residue presence since fat or poorly vascularized tissue would have a slower absorption rate than skeletal muscle (Shelver et al, 2014)

Withdrawal times for residue depletion in the kidney could not be calculated since they did not meet inclusion criteria outline in the FDA Guidance (FDA, 2006). The only tissue that met that requirement was the injection sites for both treatment groups. The 6 mg/kg dose only had one sow at day 1 with a detectable residue, but this point followed the linear trend and was included in the withdrawal calculation. Values for the other two animals at this time were included in withdrawal estimation at half the limit of quantification. For a dose of 6 mg/kg ampicillin trihydrate with the entire drug given at one injection site, a minimum of 41 days would be needed to assure residue depletion in 99% of the population. At the 12 mg/kg dose with a maximum injection volume of 10 mL at one injection site, 89 days would be required for injection site residue depletion. Injection sites are currently considered equal to other tissue types and their withdrawal periods are calculated identically. Injection site residue depletion tends to be much longer than other tissues due to erratic absorption of some drugs. This may artificially increase the withdrawal time for a particular drug beyond what is actually needed to meet the withdrawal period guidelines (Sanquer et al, 2006).

Matrices for residue testing

Plasma residue concentrations of ampicillin were closely associated with kidney tissue residue concentrations. The number of withdrawal days at which residues could be detected in the plasma was also very similar to kidney tissues. Based on our findings plasma appears to be an appropriate ante mortem sample to collect for residue testing to prevent violative residues at

slaughter. Ampicillin residue concentrations were detected in urine much longer than any other alternative matrix or kidney tissues. Since urine is a concentrated sample of what has been eliminated from the plasma over an extended period of time, it is possible that concentrations in urine could reach detectable levels for a longer period of time than in plasma. Similarly, ampicillin may concentrate in the urine after excretion through the kidneys, and since ampicillin does not accumulate in kidney tissue, more concentrated residues would be expected in the urine when compared to kidney tissue. This result comparing urine and plasma sample types is in contrast to a study designed to compare ante mortem sampling matrices for procaine penicillin G residue detection which determined urine was a more appropriate ante mortem sample than plasma, and plasma penicillin G concentrations depleted too quickly to accurately reflect kidney residue levels (Shelver et al., 2014). Oral fluids did not yield any positive results on LC-MS/MS. It can be concluded that oral fluids are not a good ante mortem sample due to lack of presence of ampicillin in this matrix.

Test kits

The KIS™ test was evaluated in comparison to kidney ampicillin residues. KIS™ test sensitivity for ampicillin trihydrate in kidney tissue is 100 ppb. This is much higher than the tolerance for ampicillin trihydrate, 10 ppb. The only positive KIS™ test corresponded to a kidney residue concentration of 945 ppb. The two other LC-MS/MS positive kidney samples in the 6 mg/kg treatment group were below the 100 ppb sensitivity of the KIS™ test to detect ampicillin residues in kidney tissue, and resulted in negative KIS™ tests. The kidney ampicillin concentrations were, however, above the 10 ppb tolerance, which shows the discrepancy in what the current screening test is able to detect and what residues are actually present in kidney

tissues. In the 12 mg/kg treatment group, 2/3 sows had kidney ampicillin residues above the 10 ppb tolerance but below the 100 ppb sensitivity of the KIS™ test, which is at a level that would not be expected to show up as positive residues on a screening test. One sow had a kidney ampicillin concentration of 142 ng/g, and the corresponding KIS™ test returned a false negative. A larger sample volume would be helpful in determining performance of the test. Using a combination of data from both treatment groups, PPV and NPV were estimated for the KIS™ test on kidney tissues, and were both quite high, increasing confidence in the accuracy of a reported test result.

The Charm MRL™ had an overall closer association with LC-MS/MS results than the Snap™ test. The Snap™ test had a high number of false positives in both treatment groups, which may indicate that it would not be an appropriate test to use for ante mortem residue detection in urine as it could return a positive result in a sow that is truly absent of ampicillin residues. The Charm MRL™ could have utility as an ante mortem test kit to use on urine samples for residue analysis as it had a high accuracy in detecting urine ampicillin residues. With a PPV of only 0.46, the likelihood of a test positive actually representing a positive animal on a Snap™ test is quite low.

Acknowledgments

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The original research in this thesis aimed to describe the tissue residue depletion profile of both procaine penicillin G and ampicillin trihydrate after administration at two different doses to cull sows. During study design, time points were selected for the ampicillin residue trial that would evaluate residues over an extended time period if ampicillin depletion was similar to evidence provided for penicillin G depletion. Upon elucidation of the results, ampicillin trihydrate residues are depleted much more quickly from kidney and skeletal muscle than penicillin G residues using dosing regimens typically used in commercial hog production. With this information, future study into ampicillin trihydrate tissue residue depletion at shorter time points is needed to better characterize the depletion rate and for calculation of withdrawal times.

Both penicillin G and ampicillin trihydrate residues were seen in very high concentrations in the injection site, resulting in extended withdrawal times for these tissues. There was significant variability in injection site concentrations for both drugs among sows at the same sampling time point. Depletion was erratic, and inconsistent results were obtained for residue concentrations. These inconsistencies may have affected withdrawal time calculation of drug the injection site. The calculated withdrawal time for the lower dose of penicillin was actually longer than the higher dose of penicillin. It was only slightly longer, but one would expect a longer period of withdrawal for a larger injection volume. Physiologically, this seems paradoxical and further analysis or more study animals may be needed to eliminate the individual variation effects observed.

Much of the variability and discrepancy seen in depletion between injection sites and other tissues may be due to the formulation and absorption properties of these two beta-lactams. As previously discussed, drug concentration, volume injected, and local tissue reaction can affect drug absorption from a tissue. Both the trihydrate formulation of ampicillin and the procaine formulation of penicillin G were designed to increase the time it takes to be locally absorbed from the tissue to increase the time the drug remains above a target concentration in the plasma. These formulations may inherently lead to extended injection site residues. Additionally, ampicillin trihydrate can be reconstituted to different concentrations, so a different concentration of drug may alter the residue depletion observed in the original research described in this thesis. Procaine penicillin G was injected at a volume of 20 mL, which is a substantial amount of substance for an area to absorb. Smith et al. (2013) determined injection pattern and volume of drug administered at one site comparing 10 mL to 20 mL did not affect residue depletion in kidneys. However, the residue depletion at the injection site was not evaluated.

The significance of drug residues at the injection site is complicated. Critics of withdrawal period calculation from injection site residue data argue that this falsely increases drug withdrawal times. Withdrawal period is defined as the amount of time from which the drug was last administered to the time when drug residues have depleted sufficiently for an animal to be slaughtered and enter the food supply. Injection sites are currently considered equal to other tissue types and their withdrawal periods are calculated identically. Withdrawal period calculations vary based on country. The United States recommends that a withdrawal should be calculated so that in 99% of animals that have been treated, no drug residues will be present with a 95% confidence level. Injection site residue depletion tends to be much longer than other tissues due to erratic absorption of some drugs. This may artificially increase the withdrawal

time for a particular drug beyond what is actually needed to meet the withdrawal period guidelines. Sanquer et al. performed an analysis of injection site withdrawal calculations using alternative methods, and determined that the conventional withdrawal calculation if injection sites grossly overestimated a withdrawal time for a drug (Sanquer et al., 2006).

A study by Sanquer et a. was conducted to determine the probability of injection site consumption to evaluate risk associated with injection site residues in the consumer food supply. They determined that the likelihood an EU consumer would eat an injection site with an antibiotic residue over the course of one year was 62.99% to 68.56%. However, the probability of consuming an injection site residue four or more days over the course of the year was less than 1%. Based on percentage of antibiotics purchased in the UN, they determined that beta-lactams are among the top three drug classes to be present in injection sites (Sanquer et al., 2006).

Additional residue analysis was performed on alternative matrices to determine their usefulness in predicting drug residue violations at slaughter when used as an ante mortem sampling method. Urine and plasma were tested in both penicillin G and ampicillin studies. Comparison with results obtained from kidney tissues indicate that either matrix had detectable residues at similar concentrations for the duration of time it took for kidney residues to be depleted for both drugs. In most instances, urine drug concentrations were higher and persisted longer than plasma or kidney concentrations. This may be due to the mechanisms of urine production over time from a concentration of waste removed from plasma, whereas the plasma samples represent a snapshot in time of circulating residues. Testing for residues by either sample time ante mortem should provide a sensitive method of residue detection in sows. Neither oral fluids nor environmental samples performed well as an alternative matrix when compared to tissue residues. This could be due to a lack of drug present in these samples, the

difficulty of extracting a drug from these samples, or a decreased stability of drug in these matrices.

Rapid test kits were used to test urine for residues to determine usefulness of these screening methods in residue detection. For both penicillin G and ampicillin studies, the Charm MRL™ was a more specific test for beta-lactam residue detection. The Snap™ test was slightly more sensitive than the Charm MRL™, but resulted in more false positive residue results. These tests are simple to perform and could easily be implemented as a form of ante-mortem residue screening on sows where beta-lactam treatment status is unknown and the goal is to avoid violative residues. This research did not examine the test kits on plasma samples. Future research into the efficacy of these test kits in accurately determining presence of beta-lactam residues in plasma would be needed before any conclusion could be draw about its usefulness in residue screening.

This original research has determined the tissue residue depletion information for procaine penicillin G and ampicillin trihydrate at typical dosing regimens in cull sows. It can be used to provide withdrawal recommendations for either of these two beta-lactams. Although there is a labeled dose for penicillin G, the 5X dose is typically used and this research can provide withdrawal information for the extra-label use that is more commonly seen in swine. Ampicillin trihydrate is not labelled for use in swine, so all use is extra-label. The doses used in this research reflect the range of doses that can be administered to cattle, which reflects potential doses that are used in a commercial swine operation. If recommendations are made using withdrawal times of injection sites, the practical utility of penicillin G or ampicillin trihydrate as a treatment for illness in sows is greatly decreased. This research will provide practitioners and producers with the knowledge to make intelligent treatment choices for their sows with goal of

protecting consumers from violative residues from entering the food supply. This research has also provided evidence for ante-mortem residue screening using urine or plasma at potential matrices for analysis and the practical ability to use rapid test kits to accomplish the goal of residue prevention. Antibiotics are an important public health concern when present in food animal products.

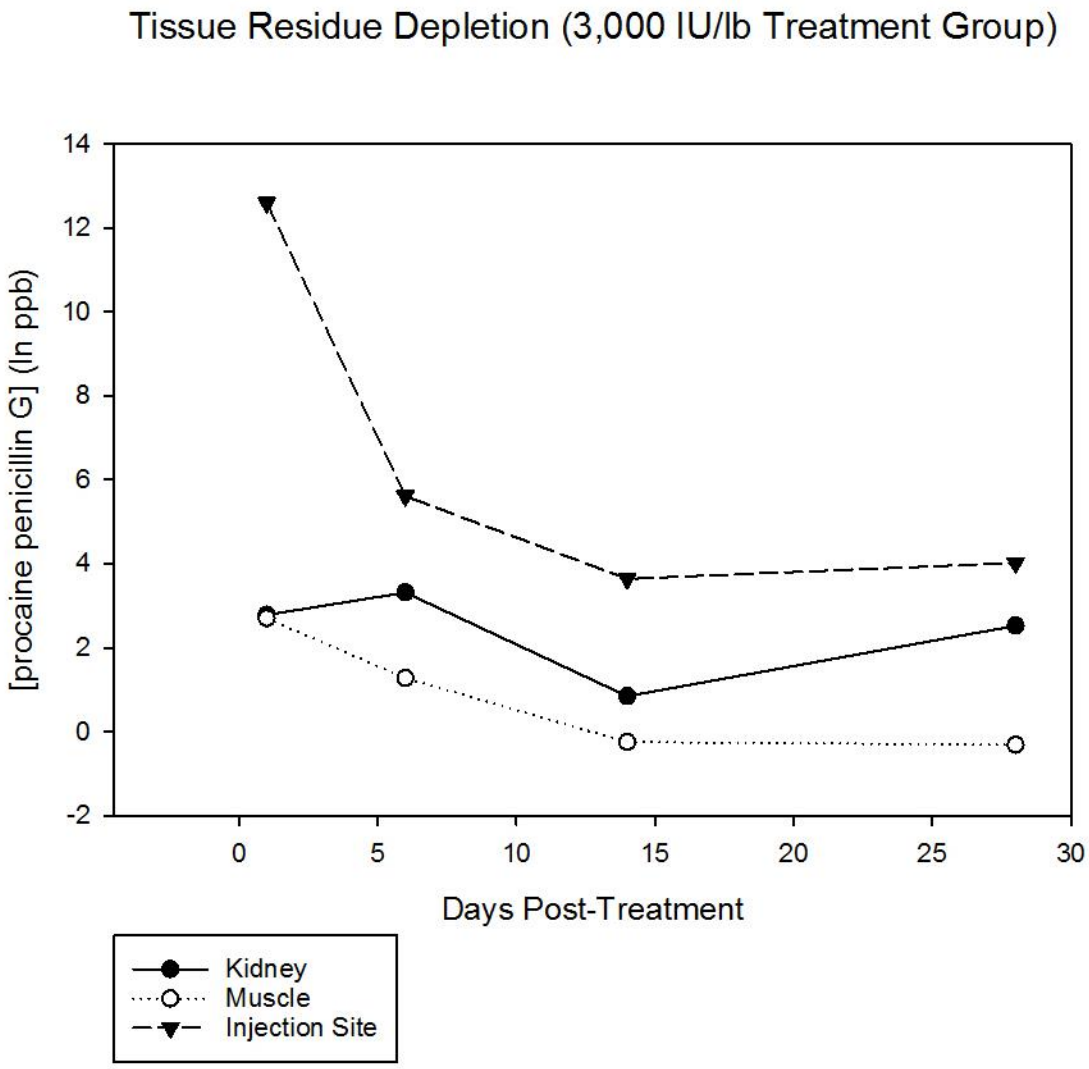

Figure 1. Tissue residue depletion of procaine penicillin G residues after intramuscular injection with 3,000 IU/lb SID for three consecutive days.

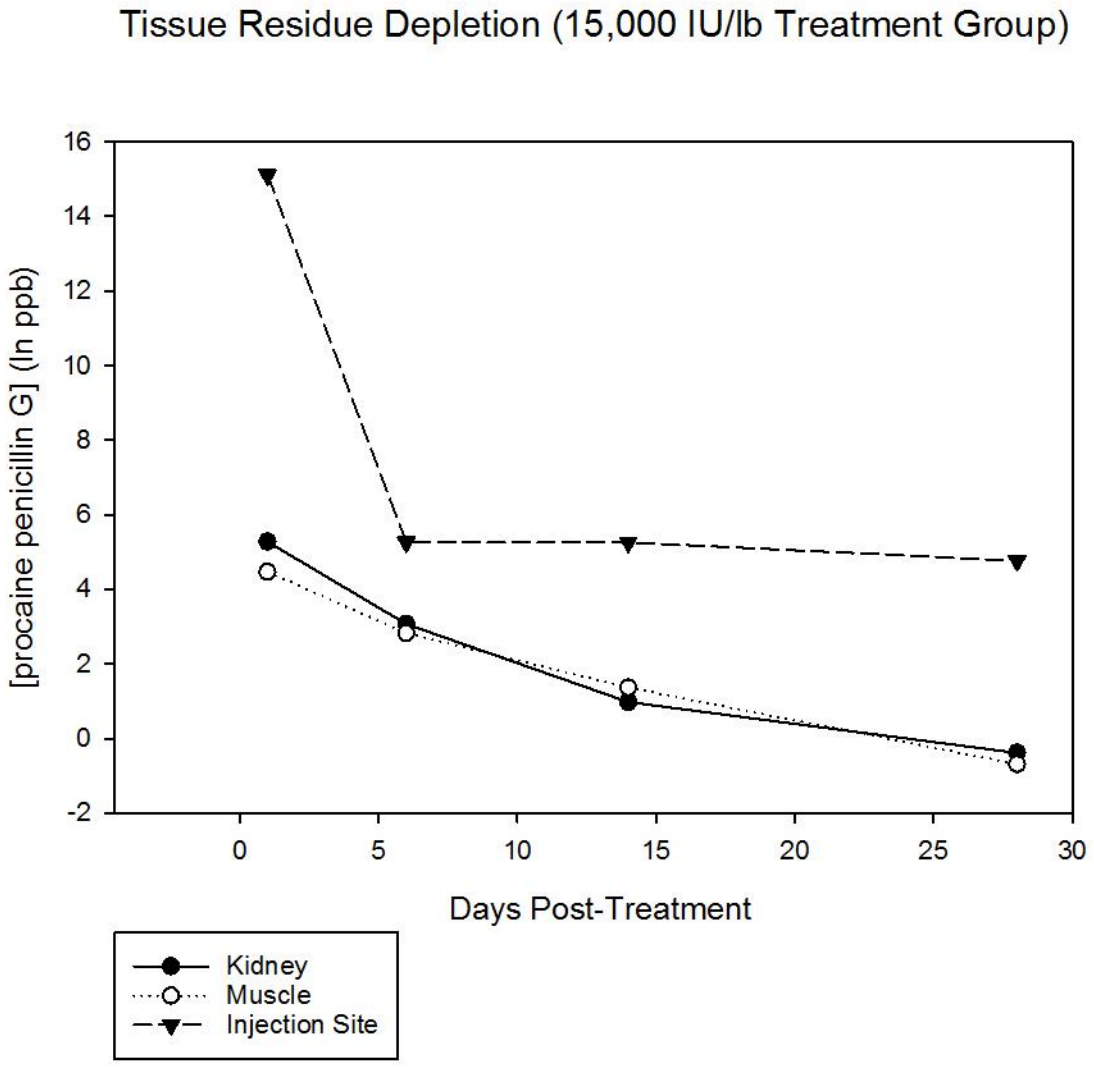

Figure 2. Tissue residue depletion of procaine penicillin G residues after intramuscular injection with 15,000 IU/lb SID for three consecutive days.

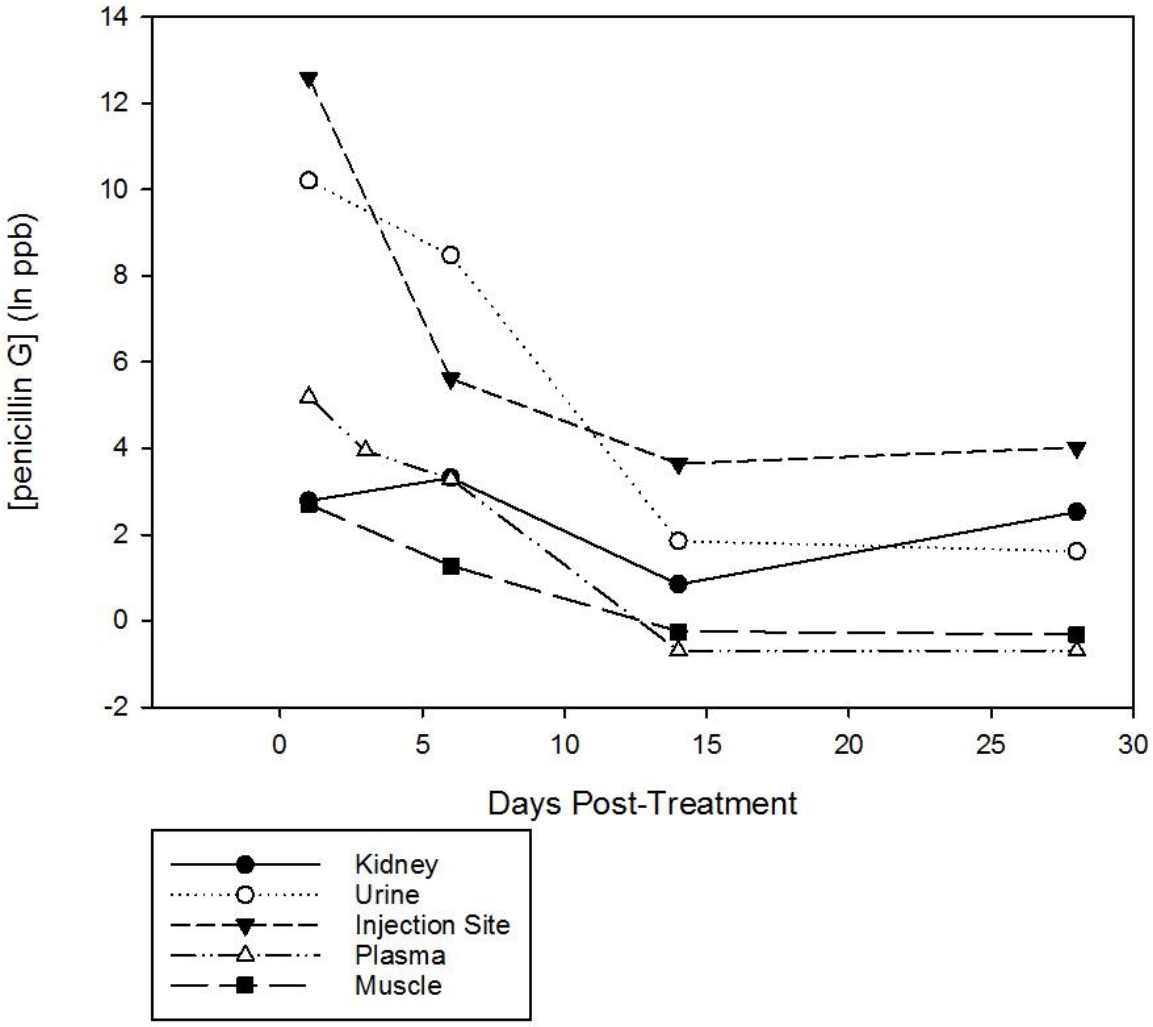

Figure 3. Comparison of mean urine and plasma residue concentrations to tissue residue concentrations over time by sampling time point for Treatment Group 1 (3,000 IU/lb procaine penicillin G IM, SID for three consecutive days).

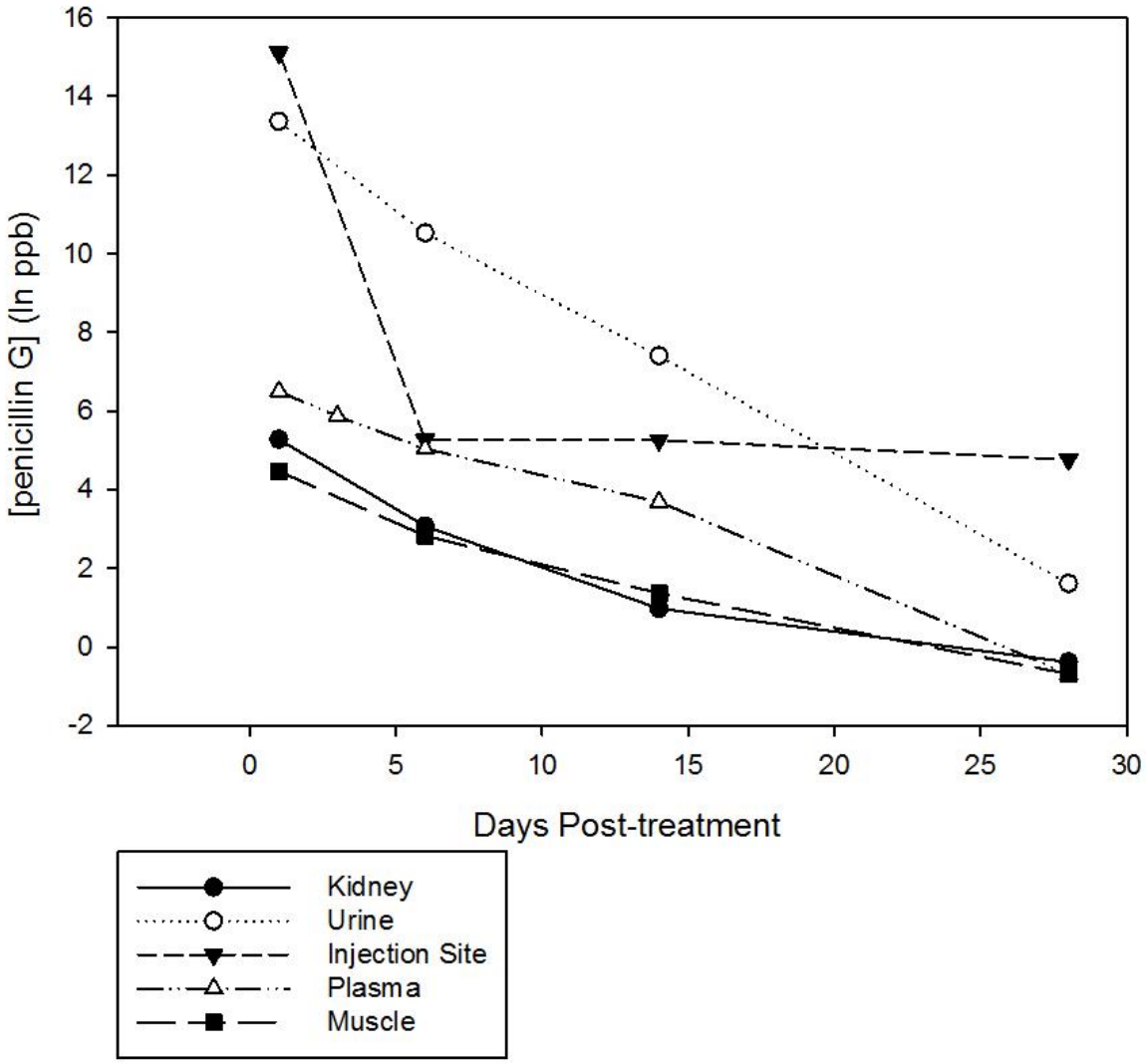

Figure 4. Comparison of mean urine and plasma residue concentrations to tissue residue concentrations over time by sampling time point for Treatment Group 3 (15,000 IU/lb procaine penicillin G IM, SID for three consecutive days).

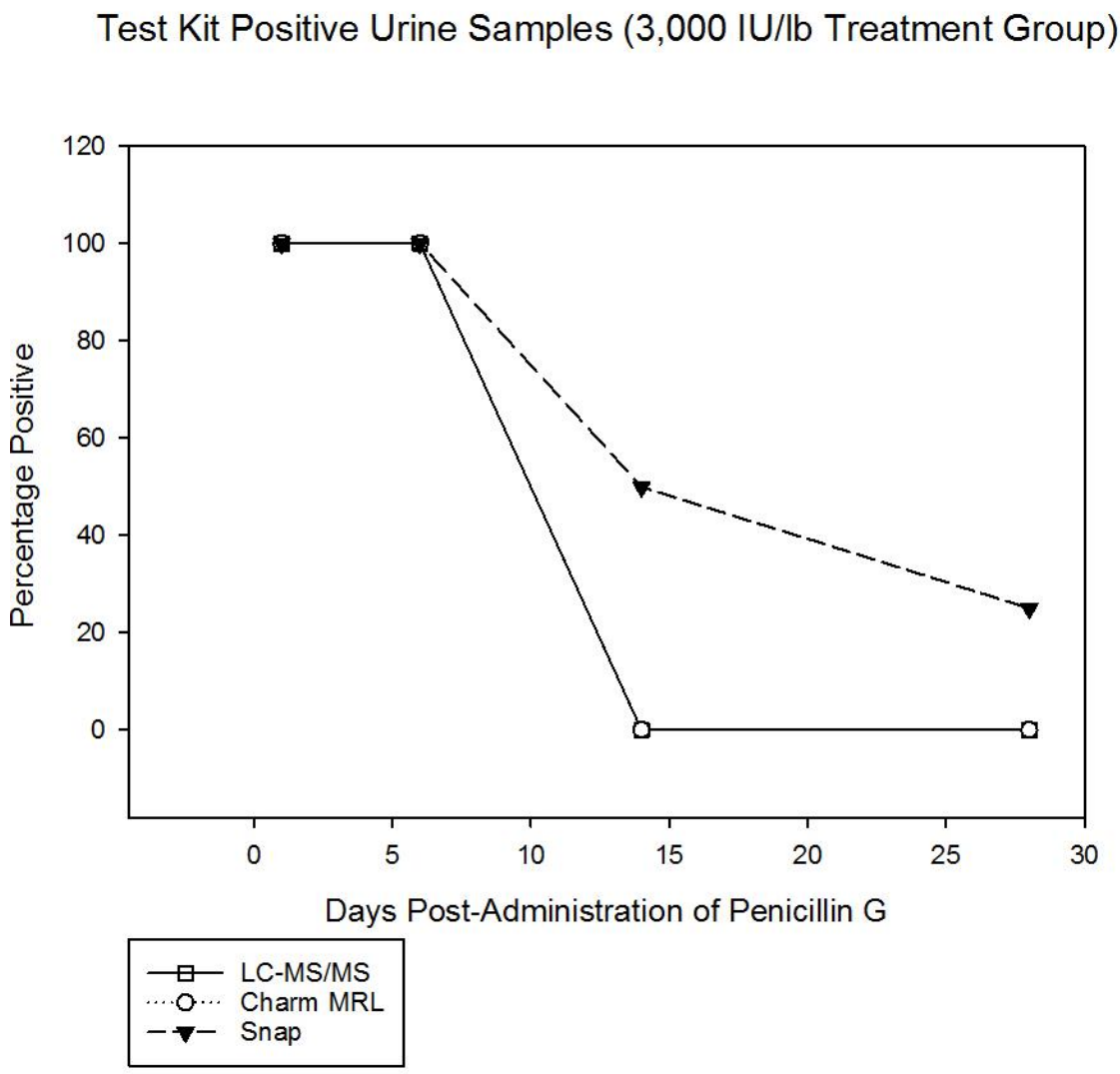

Figure 5. Comparison of Charm MRL™ and Snap™ test kits by percentage positive penicillin G residue readings to the reference LC-MS/MS percentage positive penicillin g urine residues by sampling time point for sows enrolled in Treatment Group 1 (3,000 IU/lb procaine pencillin G IM, SID for three consecutive days).

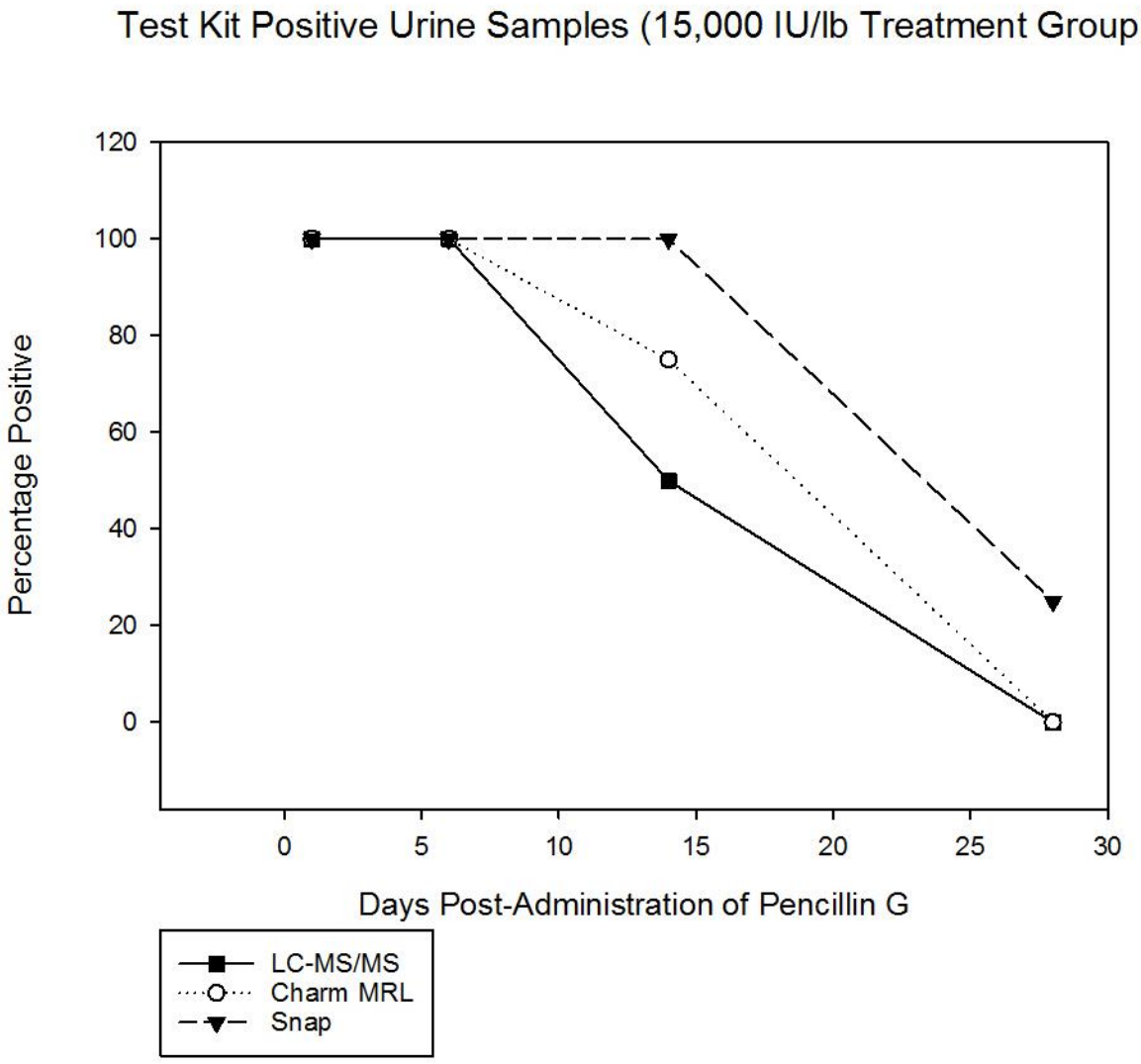

Figure 6. Comparison of Charm MRL™ and Snap™ test kits by percentage positive penicillin G residue readings to the reference LC-MS/MS percentage positive penicillin g urine residues by sampling time point for sows enrolled in Treatment Group 3 (15,000 IU/lb procaine penicillin G IM, SID for three consecutive days).

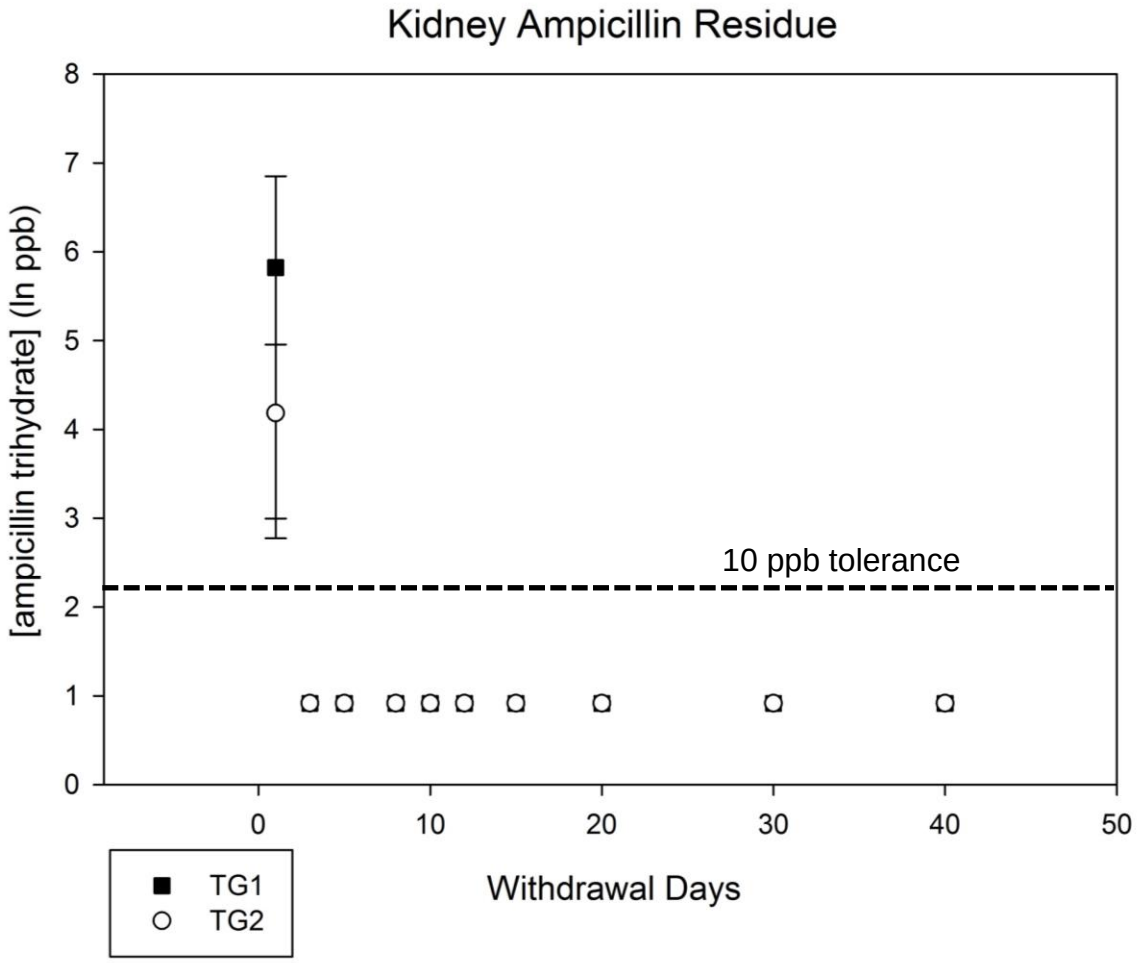

Figure 7. Kidney ampicillin residue depletion over 40 days by treatment group. Data are presented as the natural log of the mean ampicillin concentration at each time point. Corresponding concentration ranges for each time point are shown. The horizontal line represents the U. S. tolerance for ampicillin trihydrate in edible tissues of swine, 10 ppb.

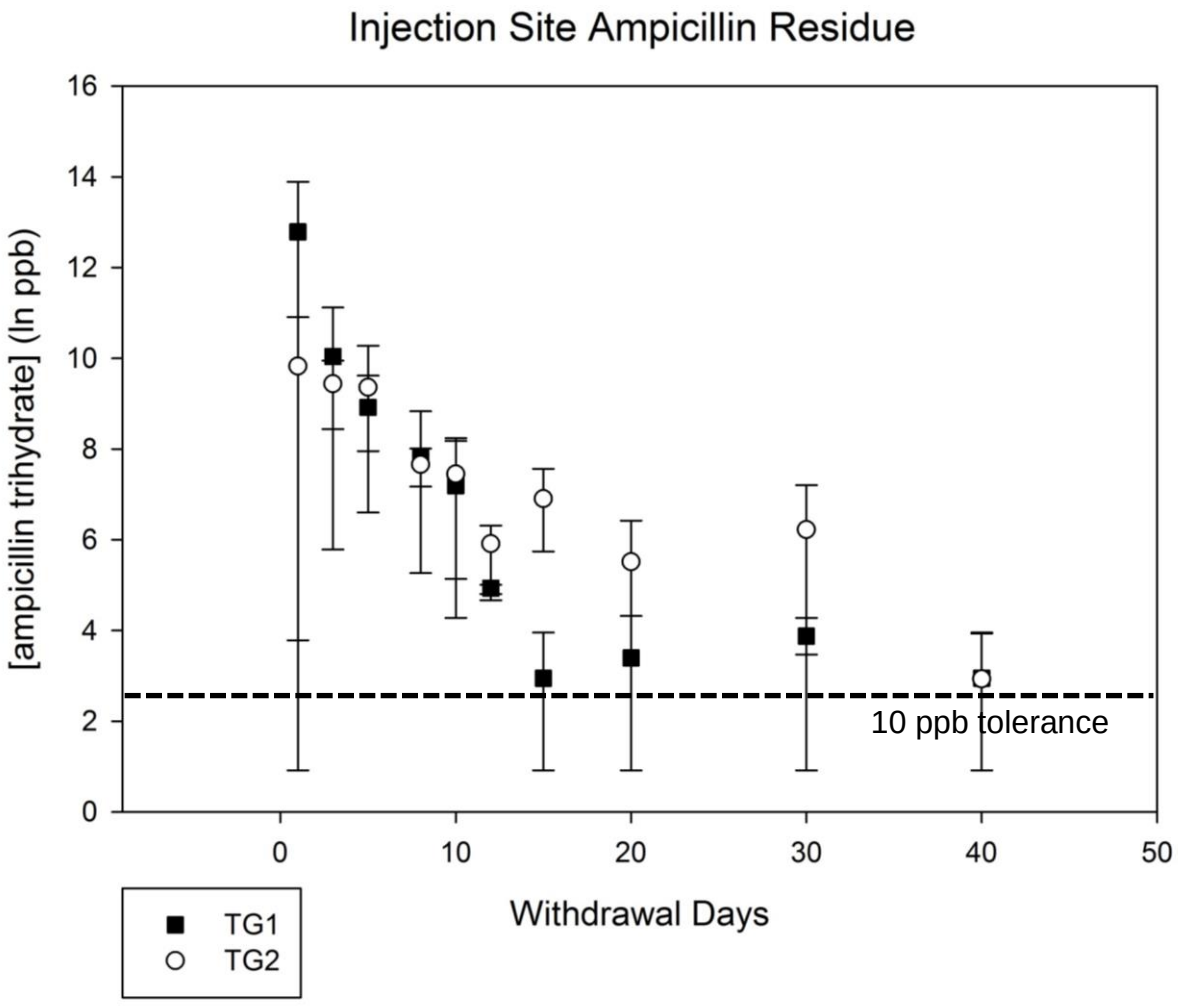

Figure 8. Injection site ampicillin residue depletion over 40 days by treatment group. Data are presented as the natural log of the mean ampicillin concentration at each time point. Corresponding concentration ranges for each time point are shown. The horizontal line represents the U. S. tolerance for ampicillin trihydrate in edible tissues of swine, 10 ppb.

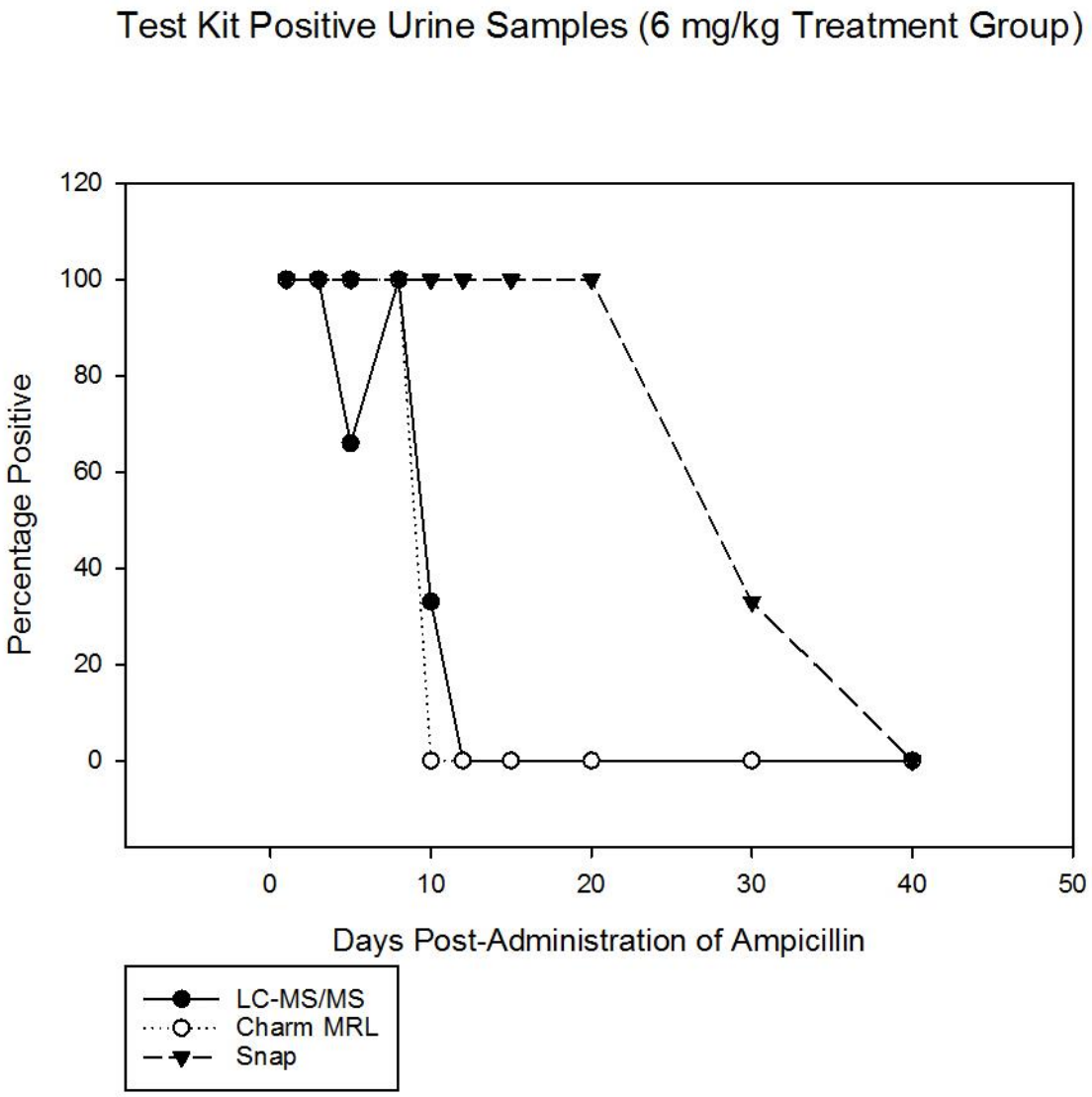

Figure 9. Comparison of Charm MRL™ and Snap™ test kits by percentage positive ampicillin residue readings to the reference LC-MS/MS percentage positive ampicillin urine residues by sampling time point for sows enrolled in Treatment Group 1 (6 mg/kg ampicillin trihydrate IM, SID for three consecutive days).

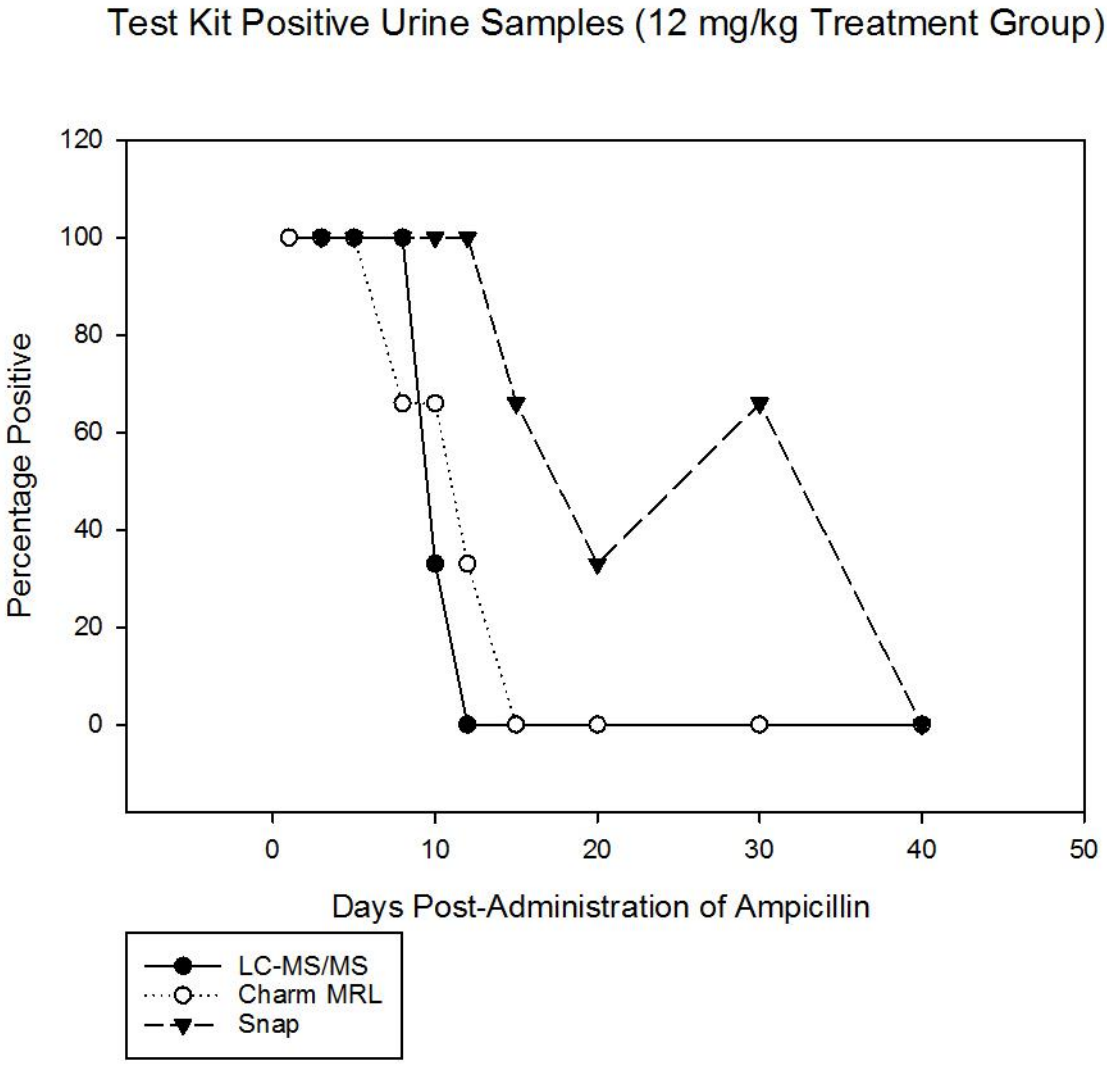

Figure 10. Comparison of Charm MRL™ and Snap™ test kits by percentage positive ampicillin residue readings to the reference LC-MS/MS percentage positive ampicillin urine residues by sampling time point for sows enrolled in Treatment Group 2 (12 mg/kg ampicillin trihydrate IM, SID for three consecutive days).

Table 1. Tissue procaine penicillin G (ng/g) in kidney, liver, semitendinosus/ semimembranosus muscle, fat, and injection site (s)

Sampling Time Point	Pen Number	Sow ID	Kidney	Liver	Muscle	Fat	Injection Site (Dorsal)	Injection Site (Ventral)
Treatment Group 1								
Day1	**1**	N/A	N/A	N/A	N/A	<LOQ	N/A	N/A
	2	342	<LOQ	<LOQ	**13.7**	<LOQ	**535872**	N/A
	3	473	**31.7**	<LOQ	**15.5**	<LOQ	**351282**	N/A
	4	339	**16.4**	<LOQ	**15.6**	<LOQ	**1151.0**	N/A
Day 6	**1**	470	<LOQ	<LOQ	<LOQ	<LOQ	**105.5**	N/A
	2	446	**96.4**	<LOQ	<LOQ	<LOQ	**895.2**	**652.2**
	3	345	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	N/A
	4	474	**11.0**	<LOQ	**6.5**	<LOQ	**97.8**	N/A
Day 14	**1**	472	<LOQ	<LOQ	<LOQ	<LOQ	**105.1**	N/A
	2	349	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	N/A
	3	344	**6.7**	<LOQ	<LOQ	<LOQ	<LOQ	N/A
	4	440	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	N/A
Day 28	**1**	340	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	N/A
	2	348	**48.4**	<LOQ	<LOQ	<LOQ	**207.7**	N/A
	3	448	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	N/A
	4	465	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	N/A
Treatment Group 2								
Day1	**1**	468	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	463	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3	462	<LOQ	<LOQ	<LOQ	<LOQ	**25.0**	**131.0**
	4	471	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Day 6	**1**	456	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	466	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3	452	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**185.0**
	4	350	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Day 14	**1**	458	<LOQ	<LOQ	<LOQ	<LOQ	**84.0**	<LOQ
	2	347	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3	451	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	4	445	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Day 28	**1**	459	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	460	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**56**
	3	343	<LOQ	<LOQ	<LOQ	<LOQ	**1124**	N/A
	4	469	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Treatment Group 3								
Day1	**1**	447	**19.4**	<LOQ	**98.2**	**12.6**	**177772**	**357204**
	2	441	**38.8**	**67.1**	**72.6**	**16.0**	**12518610**	N/A
	3	444	**44.7**	**5.8**	**115.7**	<LOQ	**1469383**	**2674478**
	4	453	**679.9**	<LOQ	**59.6**	**83.7**	**460021**	**31689**
Day 6	**1**	450	**64.9**	<LOQ	**21.7**	<LOQ	**436.5**	**3645.9**
	2	467	**16.3**	<LOQ	**19.8**	<LOQ	**227.9**	**31667**
	3	455	<LOQ	<LOQ	<LOQ	**58.8**	**53.1**	**527.6**
	4	346	<LOQ	<LOQ	**22.0**	<LOQ	**53.0**	**90121**
Day 14	**1**	443	<LOQ	<LOQ	**6.8**	<LOQ	<LOQ	**234.7**
	2	457	<LOQ	<LOQ	<LOQ	<LOQ	**85.7**	<LOQ
	3	454	**6.2**	<LOQ	**6.4**	<LOQ	**51.7**	**71.6**
	4	461	<LOQ	<LOQ	<LOQ	<LOQ	**599.8**	<LOQ
Day 28	**1**	341	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	442	<LOQ	<LOQ	<LOQ	<LOQ	**433.0**	**194.5**
	3	464	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	4	449	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 2. Penicillin G Withdrawal Times

Procaine Penicillin G Dose	Tissue	Residue Cut-off	Withdrawal time
		ppb	*d*
3,000 IU/lb	Muscle	25	8
		5	15
		1	22
	Injection Site	25	38
		5	41
		1	44
15,000 IU/lb	Kidney	25	30
		5	36
		1	41
	Muscle	25	21
		5	27
		1	34
	Injection Site	25	37
		5	40
		1	42

Table 3. Plasma procaine penicillin G concentrations (ng/mL)

Sampling Group	Pen Number	Sow ID	Days Post-Administration					
			0	1	3	6	14	28
Treatment Group 1								
Day 1	**1**	N/A	N/A	N/A	-	-	-	-
	2	342	<LOQ	**169.0**	-	-	-	-
	3	473	<LOQ	**247.5**	-	-	-	-
	4	339	<LOQ	**124.0**	-	-	-	-
Day 6	**1**	470	<LOQ	-	**23.0**	**15.8**	-	-
	2	446	<LOQ	-	**85.3**	**40.2**	-	-
	3	345	<LOQ	-	**24.5**	**7.4**	-	-
	4	474	<LOQ	-	**100.3**	**43.8**	-	-
Day 14	**1**	472	<LOQ	-	**22.7**	-	<LOQ	-
	2	349	<LOQ	-	**136.6**	-	<LOQ	-
	3	344	<LOQ	-	**20.1**	-	<LOQ	-
	4	440	<LOQ	-	**71.8**	-	<LOQ	-
Day 28	**1**	340	<LOQ	-	**42.2**	-	-	<LOQ
	2	348	<LOQ	-	<LOQ	-	-	<LOQ
	3	448	<LOQ	-	**18.2**	-	-	<LOQ
	4	465	**7.5**	-	**70.1**	-	-	<LOQ
Treatment Group 2								
Day 1	**1**	468	<LOQ	<LOQ	-	-	-	-
	2	463	<LOQ	<LOQ	-	-	-	-
	3	462	<LOQ	<LOQ	-	-	-	-
	4	471	<LOQ	<LOQ	-	-	-	-
Day 6	**1**	456	<LOQ	-	<LOQ	**8.2**	-	-
	2	466	<LOQ	-	<LOQ	<LOQ	-	-
	3	452	<LOQ	-	<LOQ	<LOQ	-	-
	4	350	<LOQ	-	<LOQ	<LOQ	-	-
Day 14	**1**	458	<LOQ	-	<LOQ	-	<LOQ	-
	2	347	<LOQ	-	<LOQ	-	<LOQ	-
	3	451	<LOQ	-	<LOQ	-	<LOQ	-
	4	445	<LOQ	-	<LOQ	-	<LOQ	-
Day 28	**1**	459	<LOQ	-	<LOQ	-	-	<LOQ
	2	460	<LOQ	-	<LOQ	-	-	<LOQ
	3	343	<LOQ	-	<LOQ	-	-	<LOQ
	4	469	**24.9**	-	<LOQ	-	-	<LOQ
Treatment Group 3								
Day 1	**1**	447	<LOQ	**641.3**	-	-	-	-
	2	441	<LOQ	**620.3**	-	-	-	-
	3	444	<LOQ	**871.9**	-	-	-	-
	4	453	<LOQ	**520.4**	-	-	-	-
Day 6	**1**	450	<LOQ	-	**336.3**	**213.1**	-	-
	2	467	<LOQ	-	**401.0**	**149.5**	-	-
	3	455	<LOQ	-	**470.6**	**29.5**	-	-
	4	346	<LOQ	-	**299.4**	**220.6**	-	-
Day 14	**1**	443	<LOQ	-	**226.0**	-	**88.2**	-
	2	457	<LOQ	-	**411.6**	-	<LOQ	-
	3	454	<LOQ	-	**713.6**	-	**61.2**	-
	4	461	<LOQ	-	**298.9**	-	**9.6**	-
Day 28	**1**	341	<LOQ	-	**277.0**	-	-	<LOQ
	2	442	<LOQ	-	**552.4**	-	-	<LOQ
	3	464	<LOQ	-	**145.9**	-	-	<LOQ
	4	449	<LOQ	-	**136.2**	-	-	<LOQ

Table 4. Urine penicillin G residue concentrations determined by LC-MS/MS

Sampling Time Point	Treatment Group 1	Treatment Group 2	Treatment Group 3
	ng/mL	*ng/mL*	*ng/mL*
Day 1	N/A	<LOQ	**1156912.8**
	12488.0	<LOQ	**654840.0**
	31502.2	<LOQ	**313796.6**
	37580.8	<LOQ	**417324.0**
Day 6	**11270.8**	<LOQ	**48974.6**
	869.2	<LOQ	**26788.7**
	2335.2	<LOQ	**16663.2**
	N/A	<LOQ	**56246.8**
Day 14	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	**5419.4**
	<LOQ	<LOQ	**1145.7**
Day 28	<LOQ	<LOQ	<LOQ
	N/A	<LOQ	<LOQ
	<LOQ	<LOQ	N/A
	<LOQ	<LOQ	<LOQ

Table 5. Kidney Inhibition Swab (KIS) Test for penicillin G residues in kidney tissues. Results were reported as positive (POS) or negative (NEG). LCMS kidney values from **Table 1** are included for comparison

		Treatment Group 1		Treatment Group 2		Treatment Group 3	
Sampling Group	**Pen Number**	**LCMS (ng/g)**	**KIS Result**	**LCMS (ng/g)**	**KIS Result**	**LCMS (ng/g)**	**KIS Result**
Day 1	**1**	N/A	N/A	<LOQ	NEG	**19.4**	POS
	2	<LOQ	NEG	<LOQ	NEG	**38.8**	POS
	3	**31.7**	NEG	<LOQ	NEG	**44.7**	POS
	4	**16.4**	POS	<LOQ	NEG	**679.9**	POS
Day 6	**1**	<LOQ	NEG	<LOQ	NEG	**64.9**	POS
	2	**96.4**	NEG	<LOQ	NEG	**16.3**	NEG
	3	<LOQ	NEG	<LOQ	NEG	<LOQ	NEG
	4	**11.0**	NEG	<LOQ	NEG	<LOQ	POS
Day 14	**1**	<LOQ	NEG	<LOQ	NEG	<LOQ	NEG
	2	<LOQ	NEG	<LOQ	NEG	<LOQ	NEG
	3	**6.7**	NEG	<LOQ	NEG	**6.2**	NEG
	4	<LOQ	NEG	<LOQ	NEG	<LOQ	NEG
Day 28	**1**	<LOQ	NEG	<LOQ	NEG	<LOQ	NEG
	2	**48.4**	NEG	<LOQ	NEG	<LOQ	NEG
	3	<LOQ	NEG	<LOQ	NEG	<LOQ	NEG
	4	<LOQ	NEG	<LOQ	NEG	<LOQ	NEG

Table 6. Summary of average sow weights at arrival by treatment group and sampling time point

Sampling Time Point		**Treatment Group**			**Mean**	**n**	**P**
		1	2	3			
d		*kg*	*kg*	*kg*	kg		
1		241.59	246.53	242.05	243.55	11	
6		247.84	240.85	225.28	237.99	12	0.8231
14		239.15	217.67	242.16	232.99	12	
28		240.34	242.56	239.49	240.8	12	
	Mean	242.27	236.9	237.24			
	n	15	16	16		47	
	P		0.845				

Table 7. Tissue ampicillin concentrations (ng/g) in kidney, liver, semitendinosus/semimembranosus muscle, and injection site(s)

Sampling Time Point	Treatment Group 1					Treatment Group 2					
	Sow ID	Kidney	Liver	Muscle	Injection Site	Sow ID	Kidney	Liver	Muscle	Injection Site "right"	Injection Site "left"
Day 1	290	**48**	<LOQ	<LOQ	**1076000**	296	**142**	<LOQ	<LOQ	**54640**	**17420**
	305	**945**	<LOQ	<LOQ	<LOQ	304	**39**	<LOQ	<LOQ	**44**	<LOQ
	307	**20**	<LOQ	<LOQ	<LOQ	306	**16**	<LOQ	<LOQ	**740**	**218**
Day 3	292	<LOQ	<LOQ	<LOQ	**67380**	280	<LOQ	<LOQ	<LOQ	**20940**	**3736**
	297	<LOQ	<LOQ	<LOQ	**1182**	281	<LOQ	<LOQ	<LOQ	**4636**	**3168**
	308	<LOQ	<LOQ	<LOQ	**326**	288	<LOQ	<LOQ	<LOQ	**12044**	**1566**
Day 5	282	<LOQ	<LOQ	<LOQ	**15092**	286	<LOQ	<LOQ	<LOQ	**29100**	**8076**
	284	<LOQ	<LOQ	<LOQ	**6617**	278	<LOQ	<LOQ	<LOQ	**2852**	**842**
	300	<LOQ	<LOQ	<LOQ	**734**	289	<LOQ	<LOQ	<LOQ	**2929**	**1827**
Day 8	283	<LOQ	<LOQ	<LOQ	**193**	279	<LOQ	<LOQ	<LOQ	**1306**	**229**
	287	<LOQ	<LOQ	<LOQ	**6860**	291	<LOQ	<LOQ	<LOQ	**3012**	**2596**
	303	<LOQ	<LOQ	<LOQ	**369**	295	<LOQ	<LOQ	<LOQ	**2037**	**1015**
Day 10	298	<LOQ	<LOQ	<LOQ	**119**	293	<LOQ	<LOQ	<LOQ	**3578**	**788**
	299	<LOQ	<LOQ	<LOQ	**3795**	277	<LOQ	<LOQ	<LOQ	**1416**	**492**
	302	<LOQ	<LOQ	<LOQ	**72**	285	<LOQ	<LOQ	<LOQ	**170**	**618**
Day 12	318	<LOQ	<LOQ	<LOQ	**122**	312	<LOQ	<LOQ	<LOQ	**550**	**139**
	329	<LOQ	<LOQ	<LOQ	**149**	314	<LOQ	<LOQ	<LOQ	**451**	<LOQ
	336	<LOQ	<LOQ	<LOQ	**145**	322	<LOQ	<LOQ	<LOQ	**106**	**111**
Day 15	317	<LOQ	<LOQ	<LOQ	<LOQ	316	<LOQ	<LOQ	<LOQ	**747**	**205**
	320	<LOQ	<LOQ	<LOQ	<LOQ	323	<LOQ	<LOQ	<LOQ	**1937**	**66**
	327	<LOQ	<LOQ	<LOQ	**52**	335	<LOQ	<LOQ	<LOQ	**311**	<LOQ
Day 20	311	<LOQ	<LOQ	<LOQ	**12**	321	<LOQ	<LOQ	<LOQ	**125**	<LOQ
	331	<LOQ	<LOQ	<LOQ	**75**	324	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	332	<LOQ	<LOQ	<LOQ	<LOQ	334	<LOQ	<LOQ	<LOQ	**614**	**105**
Day 30	319	<LOQ	<LOQ	<LOQ	**72**	315	<LOQ	<LOQ	<LOQ	**130**	<LOQ
	325	<LOQ	<LOQ	<LOQ	**70**	328	<LOQ	<LOQ	<LOQ	**1348**	**208**
	330	<LOQ	<LOQ	<LOQ	<LOQ	333	<LOQ	<LOQ	<LOQ	**32**	**28**
Day 40	309	<LOQ	<LOQ	<LOQ	**52**	313	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	310	<LOQ	<LOQ	<LOQ	<LOQ	337	<LOQ	<LOQ	<LOQ	**51**	<LOQ
	326	<LOQ	<LOQ	<LOQ	<LOQ	338	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 8. Ampicillin trihydrate tissue withdrawal time

Ampicillin Trihydrate Dose	Tissue	Residue Cut-off	Withdrawal Time
		ppb	*d*
6 mg/kg	Injection Site	10	41
12 mg/kg	Injection Site	10	89

Table 9. Plasma ampicillin trihydrate concentrations (ng/mL)

Sampling Time Point	Sow ID	Days Post-Administration										
		0	1	3	5	8	10	12	15	20	30	40
Treatment Group 1												
Day 1	305	**5**	**110**	-	-	-	-	-	-	-	-	-
	307	<LOQ	**88**	-	-	-	-	-	-	-	-	-
	290	<LOQ	**118**	-	-	-	-	-	-	-	-	-
Day 3	308	**10**	-	<LOQ	-	-	-	-	-	-	-	-
	292	<LOQ	-	<LOQ	-	-	-	-	-	-	-	-
	297	<LOQ	-	<LOQ	-	-	-	-	-	-	-	-
Day 5	284	**20**	-	-	<LOQ	-	-	-	-	-	-	-
	282	<LOQ	-	-	<LOQ	-	-	-	-	-	-	-
	300	<LOQ	-	-	<LOQ	-	-	-	-	-	-	-
Day 8	283	<LOQ	-	-	-	<LOQ	-	-	-	-	-	-
	287	<LOQ	-	-	-	<LOQ	-	-	-	-	-	-
	303	<LOQ	-	-	-	<LOQ	-	-	-	-	-	-
Day 10	298	<LOQ	-	-	-	-	<LOQ	-	-	-	-	-
	302	<LOQ	-	-	-	-	<LOQ	-	-	-	-	-
	299	<LOQ	-	-	-	-	<LOQ	-	-	-	-	-
Day 12	318	<LOQ	-	-	-	-	-	<LOQ	-	-	-	-
	329	<LOQ	-	-	-	-	-	<LOQ	-	-	-	-
	336	<LOQ	-	-	-	-	-	<LOQ	-	-	-	-
Day 15	317	<LOQ	-	-	-	-	-	-	<LOQ	-	-	-
	320	<LOQ	-	-	-	-	-	-	<LOQ	-	-	-
	327	<LOQ	-	-	-	-	-	-	<LOQ	-	-	-
Day 20	311	<LOQ	-	-	-	-	-	-	-	<LOQ	-	-
	331	<LOQ	-	-	-	-	-	-	-	<LOQ	-	-
	332	<LOQ	-	-	-	-	-	-	-	<LOQ	-	-
Day 30	319	<LOQ	-	-	-	-	-	-	-	-	<LOQ	-
	325	<LOQ	-	-	-	-	-	-	-	-	<LOQ	-
	330	<LOQ	-	-	-	-	-	-	-	-	<LOQ	-
Day 40	309	<LOQ	-	-	-	-	-	-	-	-	-	<LOQ
	310	<LOQ	-	-	-	-	-	-	-	-	-	<LOQ
	326	<LOQ	-	-	-	-	-	-	-	-	-	<LOQ
Treatment Group 2												
Day 1	296	<LOQ	**471**	-	-	-	-	-	-	-	-	-
	304	<LOQ	**53**	-	-	-	-	-	-	-	-	-
	306	<LOQ	**51**	-	-	-	-	-	-	-	-	-
Day 3	280	<LOQ	-	**18**	-	-	-	-	-	-	-	-
	281	<LOQ	-	<LOQ	-	-	-	-	-	-	-	-
	288	<LOQ	-	<LOQ	-	-	-	-	-	-	-	-
Day 5	286	<LOQ	-	-	<LOQ	-	-	-	-	-	-	-
	278	<LOQ	-	-	<LOQ	-	-	-	-	-	-	-
	289	<LOQ	-	-	<LOQ	-	-	-	-	-	-	-
Day 8	279	<LOQ	-	-	-	<LOQ	-	-	-	-	-	-
	291	<LOQ	-	-	-	<LOQ	-	-	-	-	-	-
	295	<LOQ	-	-	-	<LOQ	-	-	-	-	-	-
Day 10	293	<LOQ	-	-	-		<LOQ	-	-	-	-	-
	277	<LOQ	-	-	-	-	<LOQ	-	-	-	-	-
	285	<LOQ	-	-	-	-	<LOQ	-	-	-	-	-
Day 12	312	<LOQ	-	-	-	-	-	<LOQ	-	-	-	-
	314	<LOQ	-	-	-	-	-	<LOQ	-	-	-	-
	322	<LOQ	-	-	-	-	-	<LOQ	-	-	-	-
Day 15	316	<LOQ	-	-	-	-	-	-	<LOQ	-	-	-
	323	<LOQ	-	-	-	-	-	-	<LOQ	-	-	-
	335	<LOQ	-	-	-	-	-	-	<LOQ	-	-	-
Day 20	321	<LOQ	-	-	-	-	-	-	-	<LOQ	-	-
	324	<LOQ	-	-	-	-	-	-	-	<LOQ	-	-
	334	<LOQ	-	-	-	-	-	-	-	<LOQ	-	-
Day 30	315	<LOQ	-	-	-	-	-	-	-	-	<LOQ	-
	328	<LOQ	-	-	-	-	-	-	-	-	<LOQ	-
	333	<LOQ	-	-	-	-	-	-	-	-	<LOQ	-
Day 40	313	<LOQ	-	-	-	-	-	-	-	-	-	<LOQ
	337	<LOQ	-	-	-	-	-	-	-	-	-	<LOQ
	338	<LOQ	-	-	-	-	-	-	-	-	-	<LOQ

Table 10. Urine ampicillin residues determined by LC-MS/MS

Sampling Time Point	Treatment Group 1		Treatment Group 2	
	Sow ID	LCMS	Sow ID	LCMS
		ng/mL		*ng/mL*
Day 1	290	**56200**	296	**51750**
	305	**138000**	304	**1420**
	307	**42450**	306	**156000**
Day 3	292	**87**	280	N/A
	297	**16**	281	1820
	308	**89**	288	**33**
Day 5	282	**3200**	278	**37**
	284	<LOQ	286	**20**
	300	**7**	289	**90**
Day 8	283	**18**	279	**11**
	287	**13**	291	**30**
	303	**18**	295	**8**
Day 10	298	<LOQ	277	<LOQ
	299	<LOQ	285	<LOQ
	302	**8**	293	**10**
Day 12	318	<LOQ	312	<LOQ
	329	<LOQ	314	<LOQ
	336	<LOQ	322	<LOQ
Day 15	317	N/A	316	<LOQ
	320	<LOQ	323	<LOQ
	327	<LOQ	335	<LOQ
Day 20	311	<LOQ	321	<LOQ
	331	<LOQ	324	<LOQ
	332	<LOQ	334	<LOQ
Day 30	319	<LOQ	315	<LOQ
	325	<LOQ	328	<LOQ
	330	<LOQ	333	<LOQ
Day 40	309	<LOQ	313	<LOQ
	310	<LOQ	337	<LOQ
	326	<LOQ	338	<LOQ

Table 11. Kidney Inhibition Swab (KIS™) Test for ampicillin residues in kidney tissues

Sampling Time Point	Treatment Group 1		Treatment Group 2	
	LCMS (ng/g)[†]	KIS Result[‡]	LCMS (ng/g)	KIS Result
Day 1	**48**	NEG	**142**	NEG
	945	**POS**	**39**	NEG
	20	U	**16**	NEG
Day 3	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 5	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 8	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 10	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 12	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 15	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 20	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 30	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
Day 40	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG
	<LOQ	NEG	<LOQ	NEG

‡Results were reported as positive (POS), negative (NEG), or uncertain (U).
[†]LCMS kidney values for ampicillin trihydrate concentrations from **Table 5** are included for comparison.

Table 12. Summary of average sow weights on arrival by treatment group and sampling time point

Sampling Time Point		Treatment Group		Mean	n	P
		1	2			
d		*kg*	*kg*	*kg*		
1		242.3	248.3	245.3	3	
3		241.1	236.6	238.9	3	
5		238.0	240.6	239.3	3	
8		252.7	250.8	251.7	3	
10		244.2	253.3	248.8	3	<0.01
12		197.7	206.2	202.0	3	
15		197.5	203.0	200.3	3	
20		197.1	197.7	197.3	3	
30		199.8	212.0	205.9	3	
40		204.6	189.6	197.1	3	
	Mean	221.5	223.8			
	n	30	30		60	
	P	0.7524				

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