**Pharmacokinetics of Ampicillin-Sulbactam in Serum and Synovial Fluid Samples Following Regional Intravenous Administration in the Distal Hind Limb of Adult Cattle**

THESIS

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Abstract

Objective: The goal of this study was to define the pharmacokinetics of ampicillin-sulbactam in synovial fluid and serum of the digital circulation and central venous circulation following administration as a regional intravenous limb perfusion (RLP) of the distal hind limb in cattle.

Animals: Six healthy, adult, non-lactating dairy cows with no evidence of digital infection or lameness.

Procedures: Intravenous catheters were placed in the dorsal common digital vein (DCDV) of the right hind limb and in the jugular vein; an indwelling catheter was placed in the metatarsophalangeal joint of the right hind limb in all animals. An RLP of the distal extremity was performed using a tourniquet applied at the proximal metatarsus and 1.5 g combined ampicillin-sulbactam (1g ampicillin, 0.5g sulbactam) was administered into the DCDV. Synovial fluid was collected from the metatarsophalangeal joint, and blood was collected from the DCDV and jugular vein at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 18, and 24 hours post infusion. A single blood sample was taken from the abaxial proper plantar vein (APPV) of the lateral digit of the right hind limb at 0.25 hours post-

perfusion. Synovial fluid and serum were analyzed by high-pressure liquid chromatography (HPLC).

Results: Maximum mean concentration of ampicillin in synovial fluid, DCDV, APPV and systemic circulation (± St. Dev) were 1995 (±1011), 4827 (± 1883), 5423 (± 1953) and 2.5 (± 1.6) µg/mL respectively. Sulbactam concentrations followed similar trends to ampicillin concentrations, and sulbactam concentrations remained near half ampicillin concentrations, and above during the later time points. The best fit line for the mean concentration of ampicillin in synovial fluid dropped below 8µg/mL (the CLSI breakpoint MIC for ampicillin) at 18.9 (range 15.4-24.9) hours after RLP. No adverse events were encountered throughout the duration of the study or following removal of the catheters.

Conclusions and Clinical Relevance: Both drugs reached high concentrations in digital circulation and synovial fluid of the distal limb, but central venous blood concentrations remained low. Ampicillin concentrations remain above therapeutic concentrations for common organisms in synovial fluid for greater than 80% of a 24 hour period, without a relative decrease in sulbactam, suggesting that once daily dosing as an RLP may be sufficient to provide therapeutic concentrations to synovial structures of the bovine distal limb. Further research is needed to assess treatment efficacy in cattle clinically affected by digital infections, including deep digital sepsis.

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# Chapter 1: Introduction and Background

## 1.1 Introduction

Infections of the foot are a common cause of lameness with economic and welfare concerns in both dairy and beef cattle[1,2]. Injection of an antimicrobial directly into the digital circulation as a regional limb perfusion (RLP) can be an effective method of treating localized infections of the limb in large animals[3–5]. This technique involves tourniquet application proximal to the region being perfused, and administration of drug into the isolated circulation. This technique allows for high concentrations of drug within the isolated region while limiting systemic concentrations. This treatment modality is commonly used in a clinical setting for care of cattle with digital infections and can be applied in a field setting with appropriate restraint. Two common bacterial pathogens associated with infections of the bovine foot are *Fusobacterium necrophorum* and *Trueperella pyogenes;* both of which have demonstrated susceptibility to ampicillin.[6–10] Ampicillin is a β-lactam antimicrobial and sulbactam is a drug that inhibits destruction of β-lactam antimicrobials by β-lactamases (bacterial enzymes that degrade β-lactam antibiotics). This antimicrobial is a time dependent antibiotic; efficacy depends on the length of time the drug concentration remains above a Minimum Inhibitory Concentration (MIC)[11]. Ampicillin has been used clinically as an RLP for the treatment of deep digital sepsis, however no data is available in the peer reviewed literature to

describe the pharmacokinetics of this drug when used as an RLP in cattle[12].

Antimicrobial usage in food animals is under increasing scrutiny, and the importance of using evidence- based medicine when treating cattle with antimicrobials cannot be overemphasized. The study described herein documents the concentration versus time curve of the drug combination ampicillin-sulbactam in the digital circulation and synovial fluid of cattle, when used in cattle as an RLP. This curve can be compared to published MIC data for common bacterial pathogens of the bovine distal limb to provide targeted information to guide treatment intervals and evidence-based patient care.

## 1.2 The problem of bovine lameness and deep digital sepsis

Lameness of the bovine foot is a common problem[13–15]. Most lameness in cattle can be localized to the digits, and further localized to the lateral claw in the rear and medial claw in the thoracic limb as these are the primary weight bearing claws in cattle[14,16]. Infectious causes of lameness are an important subset of disease and these include primary infections, as well as any disease that breaks down the normal barrier between the horn or dermis and leaves the deeper structures vulnerable to infection. Potential septic foci within the deep tissues of the bovine digit include septic arthritis (both interphalangeal joints, navicular bursa and the metatarsophalangeal joint), tenosynovitis (the flexor tendon sheaths, and/or flexor tendons) and septic pedal osteitis or osteomyelitis (phalanges or navicular bones)[16–18]. These deep infections of the distal extremity are often referred to as deep digital sepsis (DDS) and may be primary or secondary[19–21]. Lesions associated with DDS are some of the most severe and painful

causes of lameness[22–24]. The etiology of these lesions include penetrating foreign bodies, lacerations involving the deeper structures of the bovine digit, or hematogenous spread. The latter most often affects neonates rather than adult cattle. In adult cattle, however, deep infections of the bovine foot are most commonly secondary to extension of disease from soft tissue infections such as interdigital necrobacillosis (foot rot), damage to the sole including sole ulcers and sole abscesses, and damage to the horn such as hoof wall cracks and laminitis.[12,25,26]

As discussed previously, DDS in adult cattle occurs commonly secondary to extension of disease in the foot that breaks down the normal protective barrier of the hoof and sole horn. *Trueperella pyogenes* is a gram positive facultative anaerobic bacteria commonly present in many types of purulent infections of cattle[7,8,20]. It is also the most common bacteria isolated from cases of septic arthritis and DDS in cattle[27,20]. Due to the high likelihood of the presence of *T. pyogenes* in cases of DDS, this organism is the most important to consider when selecting an antimicrobial for RLP in adult cattle. *Fusobacterium necrophorum* is another common pathogen of bovine digital tissues, and the proximate cause of interdigital necrobacillosis. This is a gram negative anaerobic bacteria that causes damage to the protective external tissues of the digit. As such, *F. necrophorum* is associated with DDS and is also an important target for therapeutic intervention in clinical cases.

Other pathogens have been isolated from cases of DDS in cattle; however, *T. pyogenes* and *F. necrophorum* are the most important and most frequently isolated

bacteria in cases of adult DDS and are thus the target of initial antimicrobial treatment, unless bacterial culture and susceptibility testing suggest otherwise.

## 1.3 Treatment options for deep digital sepsis

Goals of therapy for DDS include debridement of the lesion, pain management and provision of antimicrobial therapy. Administration of antimicrobials to the affected tissues in DDS is an important adjunct treatment to debridement and can be achieved using various techniques[13]. Antimicrobials can be administered systemically or locally. Multiple antimicrobials are labeled for systemic use in cattle for the treatment of foot rot, for example ceftiofur, tulathromycin, oxytetracycline and florfenicol are all labeled for this purpose and thus could be rationally used for treatment of DDS. However, there are several advantages to local over systemic administration of antimicrobials for the treatment of DDS. Regional drug administration provides high local concentrations of drug compared to systemic concentrations[28–34]. These high regional concentrations may allow for drug penetration to more poorly perfused or diseased tissue[35]. Providing high local drug concentration directly to the circulation of the affected region allows for less drug to be used than would be necessary if administering the medication systemically. Limiting systemic exposure to antimicrobials is desirable to decrease meat and milk levels of antimicrobials in cattle, and thus decrease withdrawal times and potential for antimicrobial residues in the food supply. Additionally, this allows a decrease in the total dose of drug used thus limiting potential systemic side effects and decreasing the cost of treatment. Techniques for local administration of antimicrobials include intra articular

(IA) injection, intraosseous injection (IO) use of antimicrobial-impregnated implants, local infusion devices or RLP[3–5,12,36–42]. Intra-articular injection of antimicrobials can be challenging and could potentially result in introduction of pathogens into the region particularly when peri-articular cellulitis is present, and may also result in a chemical synovitis; however, RLP via local venous access avoids necessity of direct synovial access[22]. Other advantages of RLP over other methods of local drug administration include the relative ease of the technique and lack of necessity of specialized equipment, such as the bone cannula needed for IO perfusion, biocompatible antibiotic impregnable material needed for local implants, or specialized catheters and ongoing maintenance needed for indwelling infusion devices. Gentamicin impregnated sponges and beads have been described for treatment of foot infections in cattle but these treatments risk extended meat withdrawals and there is a voluntary ban on the use of aminoglycoside antibiotics in the US food animal industry[43–45]. Necessary equipment for clinical application of an RLP requires only a safe way to restrain the animal as would be routinely used for digital examination, a tourniquet, and intravenous injection supplies. This procedure is generally well tolerated by large animals, whereas the pressures necessary for IO perfusion may cause significant pain necessitating sedation or anesthesia during infusion; additionally, this procedure is associated with an increased risk of complications[5,29,31,46].

1.4 Drug selection for regional limb perfusion and extra-label drug use in food animals

Many factors are considered when selecting an appropriate antimicrobial for use in an RLP in cattle. The drug should be efficacious against the target organism(s). This information is most accurately assessed by results of bacterial culture and sensitivity; however, such information is not immediately available to the clinician. While awaiting culture and antimicrobial susceptibility results, therapy can be based on knowledge of the disease process and clinical suspicion of the most likely offending organism or organisms. In the case of DDS in adult cattle, *T. pyogenes,* and *F. necrophorum* should be the major targets of initial antimicrobial therapy[20]. The selected drug should also be safe to give IV, and should reach adequate concentrations at the site of infection. Drug concentrations should be greater than the MIC of the target organism for at least half of the dosing interval for time dependent antimicrobials such as those in the β-lactam class[11,47]. The drug should not violate drug use regulations or leave prohibitive residues.

There are no drugs approved for use as an RLP in cattle, so consideration of off label drug use is necessary. Multiple classes of antimicrobials are precluded from use as an RLP due to drug use restrictions that prohibit off label use of these classes of antimicrobials in food-producing species (including vancomycin, fluoroquinolones, cephalosporins, and sulfonamides). Extended meat and milk withdrawal times following use of some classes of antimicrobials, including aminoglycosides and macrolides, makes them less ideal for use in RLPs in the bovine[45,48,49]. The pharmacokinetics of vancomycin, gentamicin, ceftiofur, erythromycin, amikacin, amphotericin B, chloramphenicol and marbofloxacin have been studied in horses[32–34,50–56]. Tetracycline,

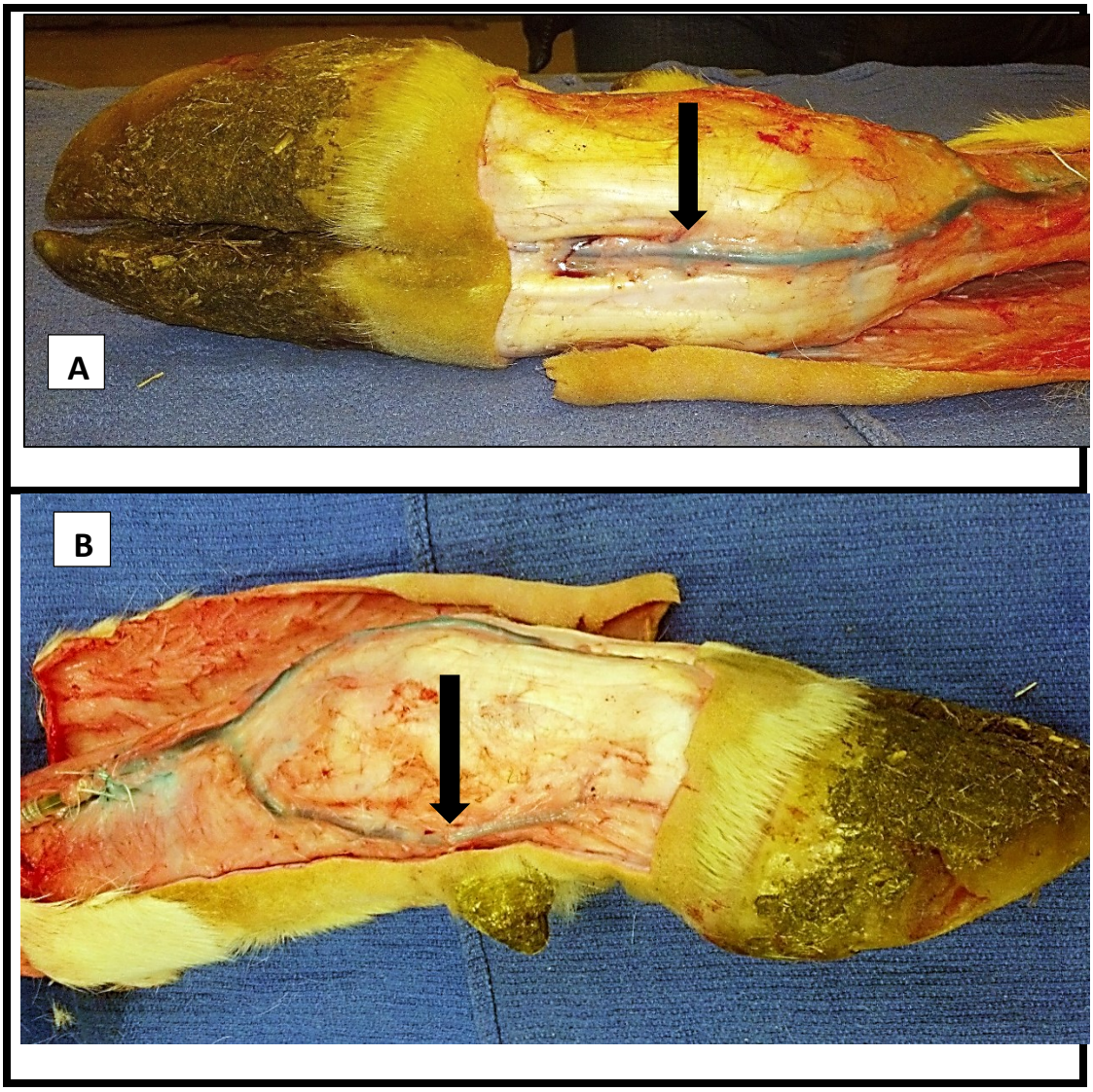
ceftiofur, cefazolin, and florfenicol have been studied in cattle[28–31]. Although ceftiofur is labeled for use in cattle, including for the treatment of foot rot, it is not labeled for IV use. As of 2012, the FDA restricted the use of cephalosporins to the labeled dose, route and frequency; as RLP requires IV administration, such use would be considered an off label route and thus not legal in the US[48,49]. The use of compounded tetracycline HCl is illegal in the US under the Animal Medicinal Use Clarification Act of 1994 (AMDUCA)[48,57]. Florfenicol carries an extended meat withdrawal, and is not labeled for use in lactating dairy cattle; and in dairy cattle, any drug that is not approved in lactating dairy cattle is considered an unapproved drug. Thus, any use of this drug in a lactating dairy cow resulting in antimicrobial residues being present in either meat or milk would be considered a violative residue by the FDA. Therefore, few antimicrobial drugs have been studied that can be safely administered via RLP in lactating dairy cattle and that do not have an extended meat withdrawal time. Ampicillin has been used clinically for the treatment of deep digital sepsis; however no data are available in the peer reviewed literature to describe the pharmacokinetics of this drug when used as an RLP in cattle[12].

# Chapter 2: Literature Review

## 2.1 Regional intravenous limb perfusion in cattle

The vasculature of the bovine distal limb has been described in detail at the gross and microscopic level[58,59]. Three veins drain blood from the digits, the dorsal common digital vein and the axial and abaxial proper plantar veins and these will be discussed in the context of the rear limb[58]. The *digitalis dorsalis communis* (dorsal common digital vein, or DCDV) is the large common vein running dorsally between the digits that forms at the junction of the dorsal proper digital veins supplied by each claw. The axial and abaxial proper plantar digital veins originate from the venous plexuses of the dermis, axial wall and bulb of their respective claws and run axially and abaxially, respectively, dorsally along each digit. Backflow is limited by the common presence of venous valves in the medium and large caliber veins and venous plexuses of the periople, coronary margin, sole and bulb[58]. The DCDV and abaxial proper plantar vein (referred to hence forth as the APPV) are easily accessible after tourniquet placement proximally on the limb, at the mid metatarsus. This anatomy allows for relative ease of access to the digital circulation in the bovine. This DCDV is illustrated in a cadaver limb in Figure 1, Panel A, and the corresponding APPV is illustrated in panel B.

Figure 1: Cadaver Distal Extremity with Venous Contrast to Illustrate Vasculature




Panel A: arrow= dorsal common digital vein; Panel B: arrow = abaxial proper plantar vein

An RLP is performed by temporarily reducing peripheral blood supply to, and venous return from, the distal extremity via tourniquet application, during which time the drug is administered in a local venous site. This technique was initially described in 1908 by Bier, who used regional perfusion for local anesthesia in humans; in 1965 Antalovsky

modified the technique for use in cattle[60,61]. This basic technique is still widely used today in food animal medicine for both regional anesthesia and regional antimicrobial administration[14,62]. Occlusion of blood flow via application of a tourniquet to the metatarsus or metacarpus allows for local diffusion of pharmaceuticals that are administered in the DCDV[28–31]. Both venous and arterial circulation should be occluded to limit blood flow during the perfusion; the pressure required to achieve successful occlusion and limit leakage during RLP has been suggested to be at least 300mmHg.[63] The success of vascular occlusion may vary depending on the type of tourniquet used for this purpose. In humans, a tourniquet at least 20% wider than the diameter of the limb is recommended[53,63,64]. Levine et al found that pneumatic tourniquets and wide rubber tourniquets were appropriate for use above the carpus in the equine thoracic limb, and that narrow rubber tourniquets were not sufficient[64]. Alkabes et al found that an Esmarch tourniquet (a type of wide rubber tourniquet) was superior to a pneumatic tourniquet when placed at the proximal metacarpus for a similar purpose in horses[53]. Diffusion from the injection site to surrounding tissues is mediated by high local concentration gradients, as well as anatomic changes that take place under RLP injection conditions[36]. The occurrence of anatomic changes during RLP has been described in the human literature; punch biopsies were used to examine tissues before and after a high pressure RLP[65]**.** These authors concluded that RLP induced a rise in venous pressure that resulted in dilation of venous capillaries and post capillary venules as well as loosening of contact between endothelial cells and widening of the spaces between endothelial cells and

pericytes. These anatomic changes enhance diffusion of and filtration of molecules into the interstitial space.

The pharmacokinetics of tetracycline, ceftiofur, cefazolin, and florfenicol have been studied for use in RLPs in cattle[28–31]. Table 1 summarizes the scientific literature examining the use of these drugs in a bovine RLP. In 1994, Gagnon et al studied the pharmacokinetic properties of cefazolin in synovial fluid of the tibiotarsal joint after regional perfusion in the bovine hind limb[28]. The investigators found that a dose of 1/14$^{th}$ that of the systemic dosage of cefazolin remained at therapeutic concentrations in the synovial fluid for at least 4 hours after RLP, which was longer than after systemic administration. They also concluded that the tourniquet needed to be in place for 30-45 minutes to achieve appropriate local drug concentrations. In 1999, Navarre et al investigated the plasma and synovial fluid distribution of ceftiofur after RLP in cattle[29]. In Navare's study, the tourniquet was placed on the metacarpus, 500mg of ceftiofur sodium was given in the DCDV, synovial fluid samples were obtained from the metacarpophalangeal joint, and venous blood samples were obtained from the APPV. The authors concluded that based on a microbial assay using synovial fluid samples, therapeutic concentrations of ceftiofur would be present for at least 5.2 hours. Due to the recent change in legislation that restricts cephalosporin use in food animals to the label dose, route and frequency, both ceftiofur and cefazolin could not be legally used as an RLP in the US today[48]. Additionally, the short duration of time above MIC for these time dependent antimicrobials would necessitate multiple treatments per day which makes them less useful for clinical application.

In 2008 Gilliam et al described the pharmacokinetics of florfenicol in cattle after RLP and found that therapeutic concentrations were reached in digital venous and synovial fluid samples[31]. That group also proposed that due to a concentration rather than time dependent type of antimicrobial activity, as well as a spectrum of activity against *Mycoplasma spp.* organisms, this antimicrobial may be potentially more useful than many other classes of antimicrobials for treating DDS in a clinical setting. Florfenicol is not specifically restricted to label usage, and the investigators of that study concluded that florfenicol usage as an RLP was unlikely to create violative meat residues if the label meat withdrawal was used; however use in adult dairy cattle could still result in violative meat or milk residues. In 2009 Rodrigues et al investigated the use of tetracycline hydrochloride given as an RLP via the "lower lateral digital or medial vein" (synonymous with the medial or lateral APPV), with a tourniquet applied just proximal to the calcaneus at the hock and collected synovial samples from an unspecified site within the tarsal joint[30]. They found that significantly higher levels of drug reached synovial fluid when given as RLP compared to systemic administration, and that therapeutic concentrations were present in synovial fluid for up to 24 hours, with lower milk residues after RLP compared to systemic administration. Tetracycline is also not restricted to label use in the US, however there are no forms of tetracycline hydrochloride available for IV injection and such a solution would have to be compounded. When following the guidelines set forth by AMDUCA, the use of a compounded product for RLP would not be allowable in cattle[57]. There are forms of oxytetracycline that are labeled for IV use in cattle that have not specifically been studied when used as an RLP. Oxytetracyline is

closely related to tetracycline HCl with similar residue distribution and antimicrobial spectrum. However, this class of antimicrobials is bacteriostatic rather than bactericidal and carries a relatively long meat withdrawal (between 22 and 28 days depending on the formulation). Also of note, the label for some formulations of oxytetracyline including the 100mg/ml IV formulation states that this medication is not for use in lactating dairy cattle.

Table 1: Summary of Scientific Literature Examining Antimicrobial Drug Administration to Cattle Using Regional Limb Perfusions and Associated Clinical Considerations

| **Drug** | **Dose** | **Perfusion site** | **Clinical Considerations** |
|---|---|---|---|
| Cefazolin[28] | 250mg/RLP | Cranial branch of the lateral saphenous vein | • Therapeutic concentrations present for 4 hours<br>• Restricted to label use |
| Ceftiofur[29] | 500mg/RLP | DCDV | • Therapeutic concentrations present for 5.2 hours<br>• Restricted to label use |
| Florfenicol[31] | 2.2mg/kg | DCDV | • Therapeutic concentrations present for 24 hours (*some organisms)*<br>• Spectrum includes *Mycoplasma* spp.<br>• Concentration dependent activity<br>• Potential for violative meat or milk residues if used in adult dairy cattle |
| Tetracycline HCl[30] | 1000mg/RLP | Lower lateral digital or medial vein | • Therapeutic concentrations present for 24 hours<br>• Use of compounded medication*<br>• Meat withdrawal of 22†-28‡days<br>• Avoid off label use in dairy cattle†<br>• Milk withdrawal of 96‡ hours |

* Tetracycline HCl; † Oxytetracycline 100mg/ml; ‡ Oxytetracycline 200mg/ml

In addition to antimicrobial administration, RLP is also commonly used to provide regional anesthesia for surgical or painful procedures on the distal extremity in cattle. Bacteremia in cattle associated with digital infections has been described following regional anesthesia, thus potentially warranting concurrent perfusion with antimicrobials when performing regional anesthesia in a bovine with deep digital sepsis[66]. The label for the ampicillin-sulbactam product used in this study[z] states that the drug can be reconstituted with 2% lidocaine before intramuscular use to decrease pain associated with injection in humans. If extrapolated to intravenous perfusion of ampicillin-sulbactam concurrently with regional anesthesia with 2% lidocaine, this is a potentially feasible modification of this technique. This modification was not specifically investigated in this study, and potentially warrants further investigation. An investigation into the effect of regional anesthesia on RLP with amikacin in horses was conducted by Mahne et al; the investigators concluded that concurrent regional anesthesia had no significant effect on amikacin pharmacokinetics when used as an RLP[52]. The ability to combine regional anesthesia with an antimicrobial during the same perfusion would greatly increase the clinical utility of this technique as the anesthetic component would allow for desensitization while ampicillin-sulbactam provides local antimicrobial activity.

Complications associated with regional intravenous perfusion of antimicrobials are generally uncommon, however repeated regional perfusion with ceftiofur sodium was suggested to be related to development of caudal vena caval thrombosis in one bovine case, high doses of benzylpenicillin used as RLP were associated with venous thrombosis

in cattle in a separate German report, and RLP was associated with secondary septic foci in foals[5,35,67–69].

## 2.2 Ampicillin-sulbactam use and pharmacology in farm animals

Ampicillin is an aminopenicillin; it is a semisynthetic, broad spectrum β-lactam antimicrobial which works by targeting the transpeptidase enzymes (also known as penicillin binding proteins) that synthesize the bacterial cell wall. This mechanism of action confers efficacy against gram positive, gram negative and anaerobic bacteria. The amino group increases efficacy against gram negative bacteria by increasing penetration of the lipid outer membrane. Sulbactam is a semisynthetic compound that irreversibly binds β-lactamases and thus inhibits this enzyme's activity[10]. This compound works synergistically with ampicillin and restores and extends the spectrum of ampicillin in the presence of β-lactamase producing bacteria[10].

Minimum inhibitory concentrations of ampicillin for *T. pyogenes* are reported within a wide range, from 0.06 µg/mL to >64 µg/mL[7]. Geurin-Faublee et al reported a high susceptibility of ruminant origin *T. pyogenes* to β-lactam antibiotics in 1993, with no organisms isolated from clinical cases being classified as resistant[70]. However, a study of *T. pyogenes* isolates recovered from the uteri of Holsteins classified a total of 86% of isolates as resistant to ampicillin alone[7]. The MIC of ampicillin for *F. necrophorum* has been described between 0.062 to 2 µg/mL and 100% of isolates susceptible at 4 µg/mL; an $MIC_{90}$ was reported as 0.125µg/mL in a recent study[8,71]. The national Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antibiotic

Susceptibility Testing (EUCAST) publish breakpoints for antibiotic susceptibility; both organizations have classified the breakpoint of susceptible bacteria to an MIC of ampicillin of 8μg/ml, based on human data. The breakpoints available for large animals are much lower; for horses it is 0.25 μg/mL, and for pigs it is 0.5 μg/mL. Breakpoints for ampicillin susceptibility are not available for cattle.

Advantages of using ampicillin-sulbactam for regional intravenous perfusion include 1. it can be safely administered intravenously in cattle, 2. there are published MIC data for these drugs against common pathogens in cattle, and 3. it is bactericidal[6–10,72–74]. Other drugs have been studied when used as an RLP in large animals and this research has found dramatic differences in local drug concentrations and half-life when compared to systemic administration[28–33,52–54]. The pharmacokinetics of the combination of ampicillin and sulbactam has been reported in ruminants including calves, sheep and goats, after systemic administration[72,75–77]. The pharmacokinetics of ampicillin alone has been studied more extensively and reported in cattle, sheep, llamas, alpacas and camels[78–83]. Studies comparing this drug combination among different ruminant species have found few significant differences in pharmacokinetics between sheep and goats and calves and sheep[72,76,77]. However, the pharmacokinetics of these drugs have not previously been studied when used as a regional perfusion in adult large animals. Regional perfusion with ampicillin has been used clinically for the treatment of deep digital sepsis in cattle, however no data is available in the peer reviewed literature to describe the pharmacokinetics of this drug when used as an RLP in cattle[12]. Due to the concern for β-lactam resistant strains of *T. pyogenes*, sulbactam, a β-lactamase inhibitor,

could be used to extend the spectrum of ampicillin to include these resistant microbes without affecting the pharmacokinetics of ampicillin[7,10,72].

Fernández-Varón et al demonstrated a moderate volume of distribution in calves of both ampicillin and sulbactam (approximately 0.2L/kg and 0.18L/kg respectively), demonstrating that both drugs can easily permeate biologic membranes[75]. Additionally, this group as well as other investigators, found that the time to peak concentration following intramuscular injection was similar between the two drugs in ruminant studies[72,75,77]. Both drugs demonstrated active renal excretion in cattle as demonstrated by clearance rates greater than glomerular filtration rate; sulbactam was excreted more slowly than ampicillin[75]. The slower excretion of sulbactam compared to ampicillin has been demonstrated in multiple studies in ruminants, resulting in higher sulbactam: ampicillin ratios[72,76]. Similar pharmacokinetic behavior of the drugs may be important for sulbactam to remain effective. It has been demonstrated that a sulbactam: ampicillin ratio of $\geq 0.5$ is necessary for sulbactam to remain efficacious in inhibiting β-lactamases in mice[10]. However, another mouse study reported that this ratio is not important; rather, the time that the drugs remain above a critical value determines efficacy[84].

The relatively rapid elimination of ampicillin after systemic administration necessitates frequent dosing intervals; Fernandez-Varon et al suggested a 5hr duration of efficacy after a single 20mg combined intramuscular dose of the drug combination (2:1 ampicillin: sulbactam) in calves, and Montesissa et al reported a period of potential efficacy of 5 hours in calves and 6 hours in sheep after similar dosing[75,72]. However, as

discussed above, other drugs have demonstrated a longer duration of therapeutic concentration in synovial fluid when given as an RLP[33,35,56]. Because ampicillin is a time dependent antibiotic, the length of time the drug concentration remains above the MIC is equally as reaching the MIC.

Ampicillin-sulbactam is an FDA approved drug, labeled for use in humans for treatment of bacterial infections. The cost of a single dose of 1g ampicillin with 0.5g sulbactam is approximately ~$5.50. This drug combination has been used systemically in cattle to treat pneumonia, and according to one study was found to be more efficacious than a combination of a β-lactam and aminoglycoside antibiotic[85]. Under FDA extra label drug use laws defined in AMDUCA, the use of ampicillin-sulbactam for RLP in cattle is allowable. According to FDA code of federal regulations, 21 CFR 556.40, "a tolerance of 0.01ppm is established for negligible residues of ampicillin in the uncooked edible tissues of cattle and in milk tissue tolerances for ampicillin in meat and milk is 0.01ppm". Withdrawal times and tissue tolerances do not exist for sulbactam; it has a similar half-life to ampicillin, but little risk for toxicity.[86] Following a single dose of 1.5 grams combined ampicillin (1g)-sulbactam (0.5g) administered as an RLP in a 1000 pound animal, meat withdrawal has been suggested by the Food Animal Residue Avoidance Databank (FARAD) to be 6 days, and milk withdrawal to be 48 hours, making this drug a potentially reasonable choice for use as a RLP in either beef or dairy cattle.[87]

2.3 Study objectives:

The goal of this study was to establish whether a single RLP of an ampicillin-sulbactam combination would reach concentrations within the deeper structures of the bovine digit above the MICs for common pathogens associated with deep digital sepsis in cattle, and to establish the length of time the concentrations remain above the target MICs. To establish this information, the concentrations of ampicillin and sulbactam were measured in synovial fluid, local digital circulation and systemic circulation after a single RLP. Synovial fluid concentrations were used as a marker of concentrations in the deeper tissues, DCDV serum for regional circulation and jugular venous samples for systemic concentrations. Additionally, the time that ampicillin concentrations exceeded a range of MICs was also determined. This information may help establish treatment intervals and allow veterinarians to provide evidence-based patient care in cattle with digital infections.

# Chapter 3: Materials and Methods

## 3.1 Animals

Six systemically healthy, non-lactating, adult Jersey or Jersey cross cows were used. Animals were free of signs of systemic illness, lameness, and digital infection on the basis of a thorough physical exam and observation during ambulation. All animals were negative for *Mycobacterium avium* subspecies *paratuberculosis* on serum ELISA. Animal weights ranged from 330kg to 449kg (mean weight 376kg). A minimum of 24 hours prior to the study, cattle were group housed in the Veterinary Medical Center at The Ohio State University and fed a mixture of grass/alfalfa hay ad libidum. Water was provided ad libidum. During the study, cattle were fed grain during procedures to facilitate handling. The amount of grain was approximately 3 kg total per cow per day. This project was approved by the Institutional Animal Care and Use Committee at The Ohio State University (protocol # 2013A00000144).

## 3.2 Animal preparation

Animals were acclimated to stalls for a minimum of 24 hours before catheter placement. At least 24 hours preceding regional perfusion, all animals had intravenous and indwelling joint catheters placed for administration of drug and sampling as

described below. Cattle were restrained routinely via hydraulic tilt chute (Figure 2) and were sedated with 20 mg of xylazine[a] intravenously immediately prior to restraint. After placement of all digital catheters, the right hind foot was bandaged to protect catheter sites (Figure 5, panel B).

Figure 2: Cow Restrained in Hydraulic Tilt Chute

3.3 Jugular venous catheters

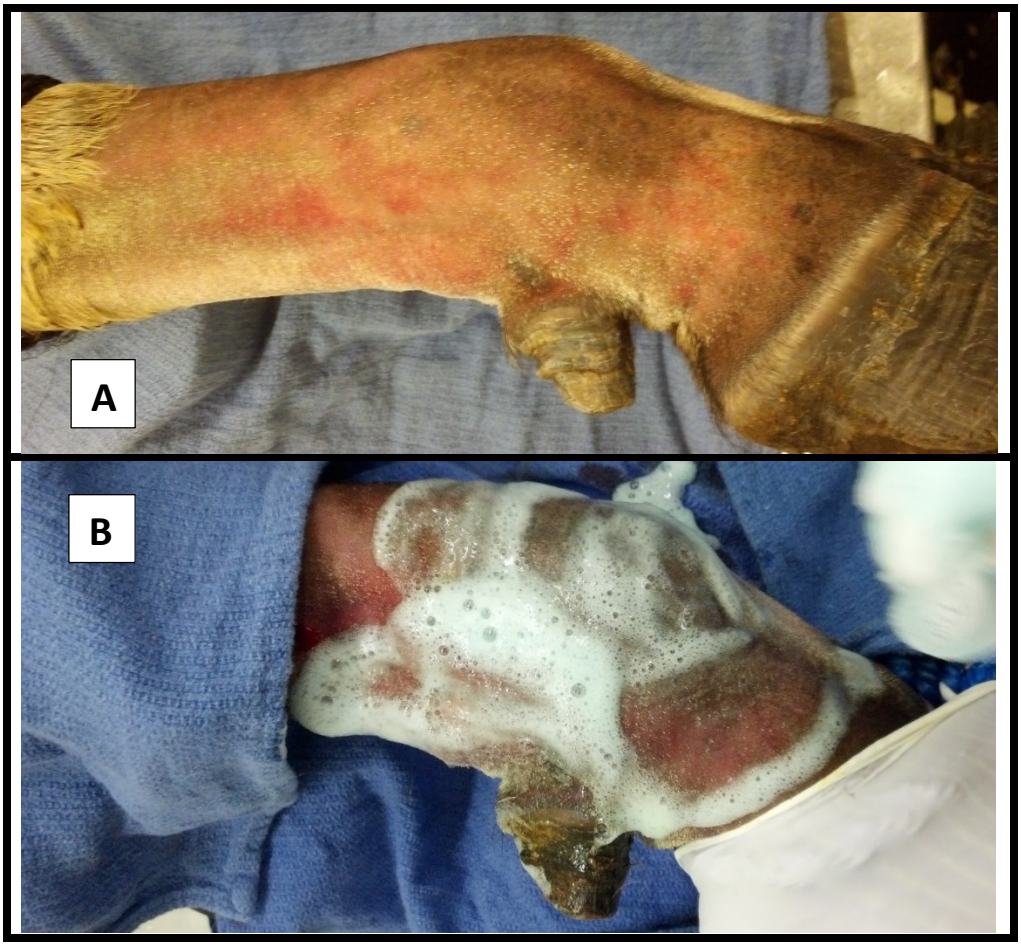
The jugular furrow on the right side of the animal's neck was clipped and aseptically prepared with chlorohexidine scrub[b] and wiped clean with alcohol[c] soaked gauze. Local anesthesia was provided by a subcutaneous injection of 2mL of 2% lidocaine[d] at the intended catheter site. A #15 blade was used to make a stab incision

through the skin. A 14 gauge 5 ¼ inch polyurethane catheter[e] was inserted into the jugular vein. A short extension set[f] and injection port[g] were secured to the end of the catheter and sutured in place with Braunamid[h] suture using a preplaced 18g needle[i]. The catheter was flushed routinely with heparinized saline[j].

3.4 Preparation of the distal limb for intravenous and indwelling joint catheters

The right hind foot was prepared as follows for catheterization of both the dorsal common digital vein and the metatarsophalangeal joint. The right hind foot was clipped from the coronary band to the mid metatarsus as shown in Figure 3, panel A and cleaned with betadine scrub[k] and wiped clean with alcohol[c] soaked gauze. A rubber Esmarch tourniquet[l] was placed around the proximal metatarsus. The digits were covered with sterile gloves. Regional anesthesia of the foot was provided via 30 ml of 2% lidocaine[d] infused in a ring block around the mid metatarsal region. The foot was then scrubbed using sterile technique with chlorohexidine scrub[b] and rinsed with alcohol[c] (Figure 3, panel B).

Figure 3: Aseptic Preparation of the Distal Extremity




Panel A: clipped distal extremity; Panel B: distal extremity during sterile scrub

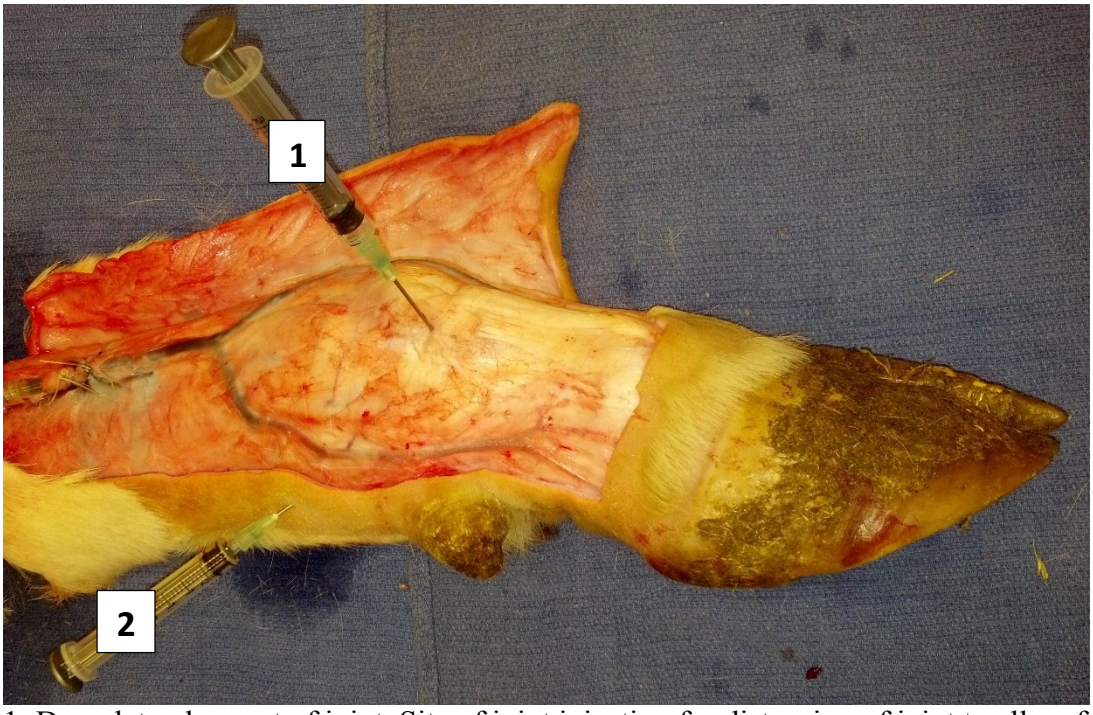
3.5 Digital catheters

3.5.1 Metatarsophalangeal joint indwelling catheters:

After aseptic preparation and local anesthesia as described above, the craniolateral aspect of the metatarsophalangeal joint was palpated. An 18 gauge 1.5 inch needle[i] was inserted at the craniolateral aspect of the joint; confirmation of placement was based on

spontaneous flow of synovial fluid from the needle. The joint was distended with 40 mL of sterile saline[m] (Figure 4, syringe 1). The caudolateral aspect of the joint pouch was palpated after distension and a 0.5 cm stab incision made through the skin with a #15 blade at the site (Figure 4, syringe 2). A 19 gauge Tuohy needle[n] was placed in the joint through this incision; pressure from joint distension was relieved by allowing excess fluid to drain. A 20 gauge polyurethane catheter[n] was placed through the needle and approximately 2 cm into the joint and the needle removed. A 16 gauge needle[o] was used to make a subcutaneous tunnel from a point 4 cm proximal to the afore mentioned incision. The other end of the catheter tubing was passed through the 16 gauge needle, and the needle was removed. The polyurethane catheter was cut 3 cm from its exit point from the skin and an injection port placed at this end and sutured in place with 2-0 polypropylene suture[p]. To further secure the injection port, white medical tape was placed around the port and the free ends of the tape were stapled to the skin using skin staples[q] (Figure 5, panel A).

Figure 4: Cadaver Limb With Needles and Syringes Illustrating Metacarphophalangeal Joint Access.




1: Dorsolateral aspect of joint. Site of joint injection for distension of joint to allow for subsequent indwelling catheter placement. 2: Palmarolateral aspect of metatarsophalangeal joint. Site of indwelling joint catheter placement.

3.5.2 Dorsal common digital venous catheters

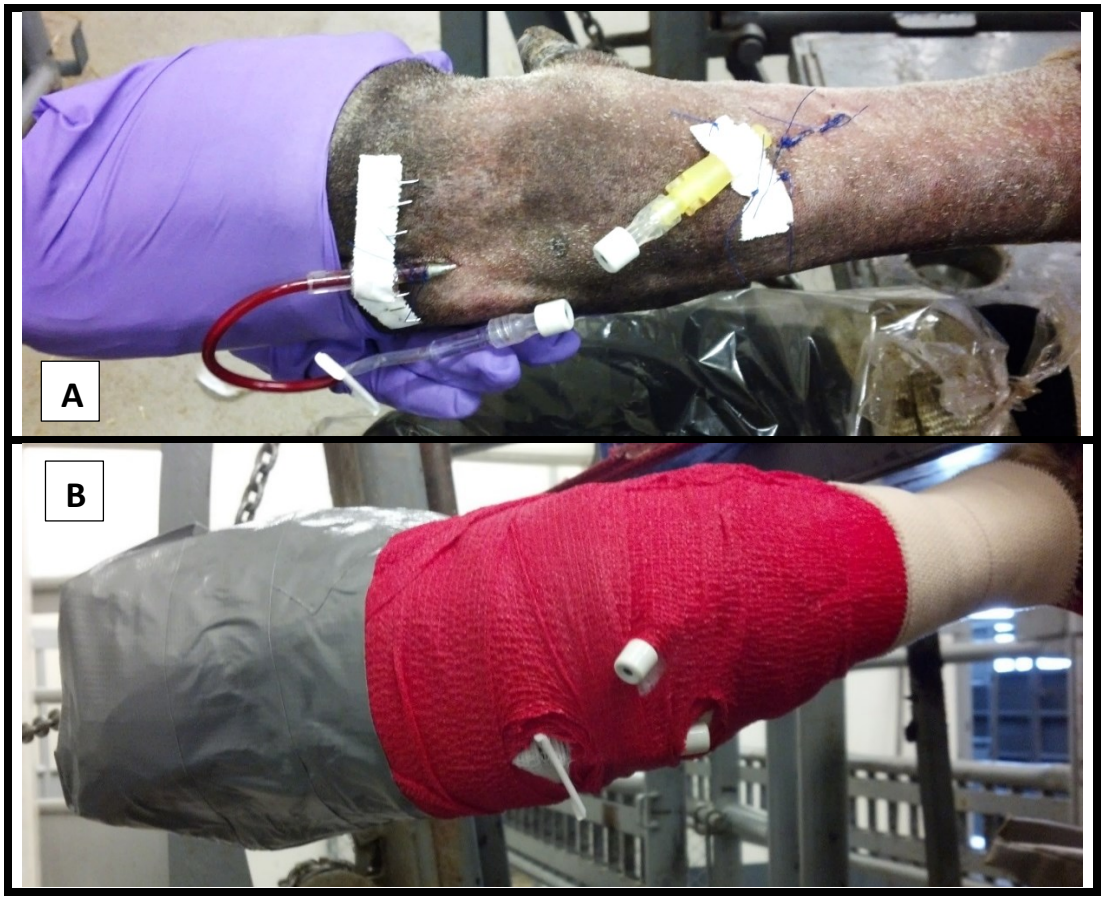
After aseptic preparation and local anesthesia as described above tourniquet in place, the dorsal common digital vein (DCDV) was palpated. A #15 scalpel blade was used to make a stab incision through the skin, over the vein approximately 2cm dorsal to the interdigital cleft. An 18 gauge needle[h] was placed in the vein. A sterile 0.018x 45cm guide wire[r] was passed through the needle and into the vein and the needle removed. An 18 gauge ETFE (ethylene tetrafluoroethylene) intravenous catheter[s] was passed over the wire and into the vein. The wire was then removed, and a short extension set[f] and

injection port[g] were placed on the catheter and sutured into place with 2-0 polypropylene suture[p] and further secured with super glue[t] (Figure 5, panel A). The catheter and extension set was heparin locked with 3000U heparin[u]

3.5.3 Bandage placement

After catheter placement, the distal extremity was bandaged to protect the catheter sites. The foot was wrapped in sterile rolled gauze[v] followed by self-adhering veterinary wrap[w], and the distal most portion was covered with waterproof adhesive tape[x] and the proximal portion secured with elasticized cotton cloth tape[y]. The catheter ports were wrapped to be available for sampling without removing the bandage (Figure 5, panel B). Finally, the catheter ports were covered in a layer of self-adhesive wrap for protection and cleanliness.

Figure 5: The Distal Extremity with Metatarsophalangeal and DCDV Catheters in Place, Before and After Bandaging.




Panel A: before bandaging. Panel B: after bandaging

3.6 Regional limb perfusion

A minimum of 24 hours following catheter placement, cattle were restrained within the hydraulic tilt chute. The outer bandage covering the digital catheter ports was removed. A rubber Esmarch tourniquet[l] was applied proximal to the digital catheter sites, at the mid metatarsal region. The tourniquet was wrapped repeatedly around the site under standard manual tension by the same investigator for each animal (KS). After

placement of the tourniquet[l], a strip of rubber tire inner tubing of similar width was wrapped in similar fashion over the tourniquet to provide stabilization and protection. Ampicillin-sulbactam[z] was prepared immediately prior to administration by adding 3.2 mL saline[m] to 1.5g combined drug (1 g ampicillin, 0.5 g sulbactam) to create a total volume of 4 mL. The entire reconstituted volume (4 mL) of combined drug was then administered via the catheter in the DCDV and flushed with 5 mL heparinized saline[j] to ensure the entire dose was delivered. In human and studies, the distal extremity is often exsanguinated prior to drug injection to increase the efficacy of the tourniquet[63]. Clinically, this could be difficult to accomplish when performing an RLP on a bovine in the field using an off the needle technique, so exsanguination was not performed in this study in order to better mimic a clinical setting. The tourniquet was left in place for 45 minutes. After 45 minutes, the tourniquet was removed, and a light bandage was applied to the foot that allowed access to injection ports. The cows were then returned to a standing position with access to food and water while sampling continued as described below.

3.7 Dosage calculations

The dose of ampicillin was based on the recommendation of 1g of ampicillin described in the literature for use as a regional limb perfusion for the treatment of deep infections of the bovine digit[88]. Additionally, a 1g dose of ampicillin is convenient because it is commercially sold in 1g sterile vials; the contents of which should be used immediately after reconstitution. The dose of sulbactam was based on the commercial

formulation that includes a 2:1 ratio of ampicillin to sulbactam, for a total of 1g ampicillin and 0.5g sulbactam; rodent studies that suggest this ratio is important for efficacy of both drugs[10]. This dosage is much lower than the 20mg/kg systemic dose of ampicillin-sulbactam used in other studies[75]. The total volume of perfusate, 4mL, was determined based on label recommendations for the smallest volume of diluent that could be added for human use. A small volume was desirable to ensure that the entire dose could be administered to all study animals without drastically increasing pressure within the DCDV or risking catheter leakage. Additionally, the limb was not exsanguinated prior to drug administration, and large volumes of perfusate can decrease the efficacy of the tourniquet[63].

### 3.8 Sample collection and handling

Samples were collected from all catheters (jugular vein, DCDV, metatarsophalangeal joint) at the following time points: immediately prior to infusion, and 0.25, 0.5, 0.75, 1, 1.5, 2, 4 ,8, 12, 18 and 24 hours after ampicillin-sulbactam infusion. The samples taken at time points up to and including 0.75hr were obtained while the cow was restrained in the tilt chute, and the tourniquet was in place. After this point, the tourniquet was removed and the cow was returned to a standing position. The cow was replaced in the restraint chute for each subsequent sample with return to a standing position and access to food and water between samples. Each jugular venous sample was taken by removing ≥6mL blood from the injection port to avoid dilution of

sample from saline flush, followed by 6mL sample blood. The catheter was flushed with 5mL heparinized saline[j] after each sample. DCDV sampling was obtained by removing 2.2mL blood from the injection port, followed by collection of 2-3 mL blood (sample) and then the catheter was flushed with 1.2 mL heparinized saline[j]. The minimum volume used for waste and flush of the DCDV was based on the total volume contained in the catheter, extension set and port which was determined to be 1.2 mL based on product descriptions. Approximately 0.5 to 1.0 mL of metatarsophalangeal joint synovial fluid was aspirated from the catheter port at each time point. A single 1-2 mL blood sample was taken from the abaxial plantar digital vein 0.25 hr post perfusion via standard phlebotomy using a 19g butterfly needle[aa] was taken from each animal to establish diffusion of drug from the admistration site of the DCDV to another vascular site, the APPV, within the same tourniquet isolated area. All venous blood samples were transferred to sterile glass blood collection tubes[bb] immediately after collection. All synovial fluid samples were transferred to plastic disposable microcentrifuge tubes[cc] immediately after collection. All samples were stored on ice in a cooler immediately after collection. Samples remained on ice for <24 hours, until blood samples were centrifuged. After centrifugation, serum was aspirated and transferred to plastic disposable microcentrifuge tubes[cc]. Serum and synovial fluid samples were then transferred to a freezer[dd] at -80°F until sample analysis could be completed.

## 3.9 Sample analysis

### 3.9.1 Ampicillin analysis

Drug concentrations of ampicillin in serum and synovial fluid samples were analysed by high performance liquid chromatography (HPLC). The method was validated using blank (control) matrix from cattle that had not received either medication. Ampicillin sodium[ee] was used as a reference standard and was dissolved in distilled water to prepare a spiking solution for calibration curve and quality control (QC) samples. The calibration curve consisted of eight fortified serum samples, plus a zero concentration (blank) sample, across a range of 0.05 - 10 µg/mL.

Serum samples were prepared by adding 300 µL serum to a solid phase extraction column[gg] after conditioning with methanol and distilled water according to the manufacturer's instructions. The sample was eluted with methanol and evaporated under a stream of air at 40°C. After evaporating to a dry residue, the sample was reconstituted with mobile phase and 30 µL injected into the HPLC system. All incurred samples, calibration curve samples and QC samples were processed in the same manner. Retention time for ampicillin was approximately 5-5.2 minutes, depending on the day's run.

Synovial fluid samples had to be treated with hyaluronidase prior to analysis. Ten µL of hyaluronidase was added to 200 µL of synovial sample, vortexed and centrifuged. 15 µL of the supernatant was injected directly into the HPLC system. For synovial fluid samples, both sulbactam and ampicillin could be detected in the same run. A calibration

curve consisted of 5 samples ranging from 10-100 µg/mL for sulbactam and 10-500 µg/mL for ampicillin, plus a zero concentration blank. Retention times for sulbactam and ampicillin were approximately 3-3.2 and 5-5.2 minutes, respectively.

Separation of peaks for both synovial fluid and serum samples was achieved at 40 °C with a Zorbax RX-C8 4.6 x 150 mm[hh] reverse phase column. The system consisted of an Agilent 1100 series quaternary solvent delivery system[hh], an Agilent 1100 series autosampler[hh], an Agilent 1200 series UV detector with ultraviolet absorbance[hh] set at 229 nm, and an Agilent OpenLAB software suite for data collection and analysis[hh]. The mobile phase consisted of 10 % acetonitrile and 90% phosphate buffer (0.05 M). The mobile phase was filtered and degassed prior to use and prepared fresh. The flow rate was 1 mL/min.

### 3.9.2 Sulbactam analysis

The HPLC conditions for sulbactam analysis in serum were the same as for ampicillin analysis in serum, except that the mobile phase was 96% phosphate buffer and 4% acetonitrile and the pH was 5.5. The assay for sulbactam in serum used a liquid phase extraction method. Sulbactam analytical reference standard[ff], purity >99%, was dissolved in distilled water to make a spiking solution. This solution was used to fortify blank synovial fluid and serum to make up a range of calibration curve samples used in the analysis. The calibration curve consisted of 6 standards over a range of 0.1 – 100 µg/mL, plus a zero concentration sample.

All incurred samples, calibration samples, and QC samples for sulbactam analysis in serum were processed in the same manner. In this method, 400 µL of the serum sample was added to a clean tube and 400 µL acetonitrile was added. The tubes were vortexed and centrifuged for 10 minutes. 500 µL of the supernatant was transferred to a clean tube and evaporated under a stream of air at 40 °C. The dry residue was mixed with 200 µL of mobile phase and 40 µL injected into the system. The retention time was 3-3.2 minutes. As described above, synovial samples were processed in a manner that sulbactam and ampicillin could be detected in the same run.

For both drugs, in each matrix, the acceptance criteria for the calibration curves was a linear range with a $R^2$ value of 0.99 or greater, and calibration samples had to be back-calculated to a concentration of within 15% of the nominal concentration. Fresh calibration curves were always prepared for each day's run. The lower limit of quantification (LOQ) was defined as the lowest concentration of analyte that could be quantified with acceptable precision and accuracy, and was established as the lowest point on a linear calibration curve that produced a signal/noise ratio of at least 6. Some incurred serum and synovial samples were higher than the upper limit of the calibration curve. These samples were diluted with mobile phase so that the concentrations were in the range of the calibration curve. A linear response for diluted samples was confirmed by testing diluted fortified samples.

## 3.10 Adverse events and monitoring

Potential adverse events were considered; these events included: metatarsophalangeal joint sepsis, septic or aseptic thrombophlebitis, and hypersensitivity reaction to the antimicrobial or the agents used for aseptic preparation of the catheter sites. Sepsis of the metatarsophalangeal joint was considered a possible side effect of joint catheterization and was monitored by assessing for clinical signs of joint infection including heat, pain or swelling of the joint and evidence of lameness in the affected limb. Thrombophlebitis was considered a possible side effect of venous catheterization and was monitored by assessing for signs of heat, pain or swelling at venous catheter sites. A recent study using similar joint and venous catheter and sampling techniques reported no adverse effects[31]. The risk of development of these side effects was limited by use of aseptic technique for catheter placement. Hypersensitivity reaction was considered a possible adverse outcome because ampicillin-sulbactam is a β-lactam antimicrobial and antimicrobials in this class have been rarely reported to cause hypersensitivity reactions in cattle[89]. Signs of a hypersensitivity reaction were monitored by assessing for swelling at injection site, development of hives, obtundation, or respiratory distress. Clinical experience with ampicillin suggests these reactions are exceedingly rare.

## 3.11 Statistical analysis

The concentration vs time curve was plotted for each animal, for each drug, at each site. These curves were used to obtain pharmacokinetic data for each animal. The

mean and standard deviation (St. Dev) of these data were reported. The mean concentration for each time point was plotted with error bars equal to +/- St. Dev. The time greater than MIC (T>MIC) was determined by solving for time when the best fit line of the terminal elimination phase of the concentration intercepts with various MIC values. The mean and St. Dev was reported for T>MIC.

# Chapter 4: Results

Data were analyzed using a pharmacokinetic software package freely available on the world wide web.[90] Data was best modeled by non-compartmental pharmacokinetic analysis, which has previously been reported for other RLP studies in large animals.[31,52,54] The concentration vs time curve was plotted for each animal, at each sampling site, for each drug; summary pharmacokinetic data were obtained from these analyses (Table 2). The mean concentration for all animals was plotted at each time point, for synovial fluid, DCDV and jugular venous samples (Fig 6). The limit of quantification (LOQ) was established for each drug in both synovial fluid and serum (Table 3).

Mean time above MIC (T>MIC) was established for a range of MICs for synovial fluid ampicillin concentrations based on the best fit line for the terminal slope of each animal (Table 4). This was represented graphically in Figure 8, where the best fit line for mean ampicillin concentrations (Fig 7) is shown with a variety of MICs. The intersection of this best fit line with a given MIC illustrates the end of T>MIC.

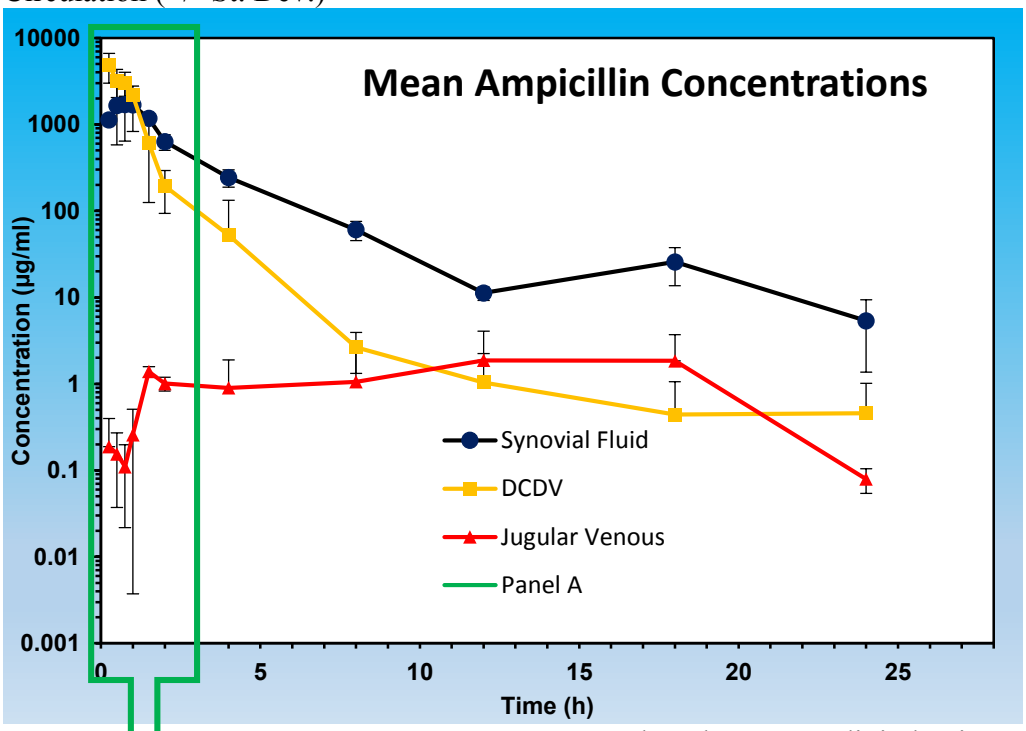
Sulbactam concentrations followed a similar trend to ampicillin (Fig 9), and retained an ampicillin: sulbactam ratio near or above 2:1 (Fig10).

Three of six cattle had no detectable ampicillin in systemic circulation by 24 hours, one animal had concentrations below the LOQ and the other two were near the LOQ (Appendix 6). Sulbactam concentrations were likewise low in systemic circulation;

in two of six animals drug was undetectable by 8 hours and below the LOQ in one animal by 8 hours; in the remaining 3 animals the mean concentration at 24 hours was near the LOQ (Appendix 5).

Study cattle tolerated the procedure and sampling well, although it should be noted that cattle displayed moderate discomfort during placement of the tourniquet and/or administration of the ampicillin-sulbactam combination, which subsided as soon as the tourniquet was removed. Because the drug was administered immediately following tourniquet placement and initial digital blood sample collection, it was difficult to determine which of these events was responsible for the apparent discomfort. No adverse events were encountered during the study; animals were monitored for at least two weeks following completion of the study during which time no adverse events were encountered.

Figure 6: Ampicillin Concentrations in Synovial Fluid, Regional, and Systemic Circulation (+/- St. Dev.)




DCDV= dorsal common digital vein

Panel A:

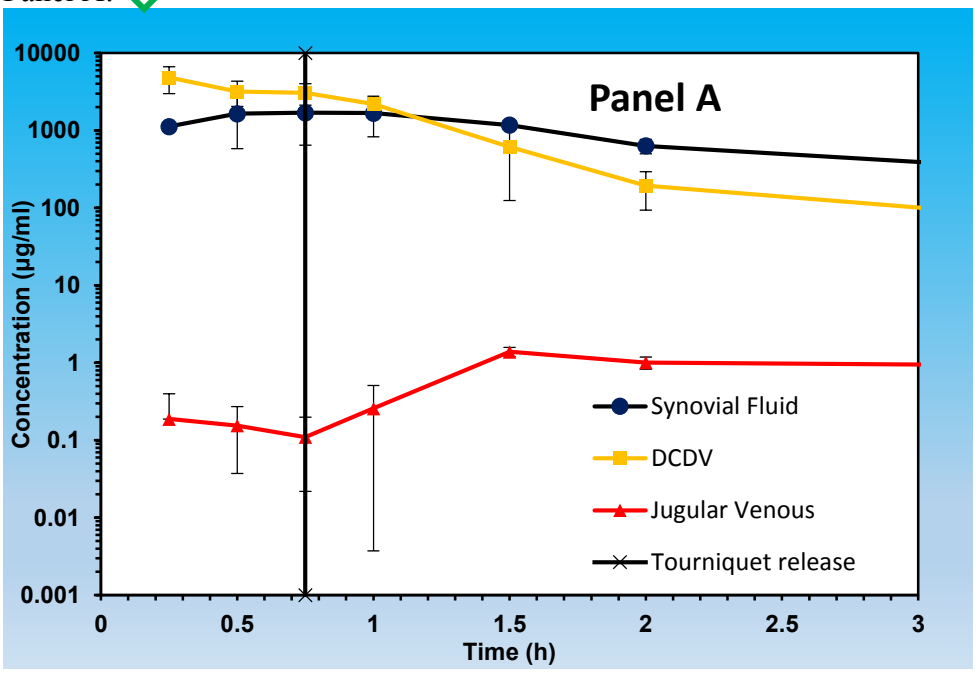


Table 2: Summary of Mean Pharmacokinetic Data (+/- St. Dev)

| **Sample Site** | **Drug** | **$C_{max}$ µg/mL** | **$T_{max}$ hr** | **$\lambda_z$ 1/hr** | **$T_{1/2}$ hr** | **$AUC_{0-t}$ µg/mL *hr** | **$AUC_{0-inf}$ µg/mL *hr** | **$MRT_{0-inf}$ hr** |
|---|---|---|---|---|---|---|---|---|
| Syn. Fluid | Amp. | 1995 (±1011) | 1 (±0.32) | 0.25 (±0.07) | 2.9 (±0.69) | 4233 (±930) | 4342 (±866) | 3.1 (±0.98) |
| Syn. Fluid | Sul. | 885 (±320) | 1 (± 0.32) | 0.39 (± 0.23) | 2.9 (±2.6) | 1823 (± 406) | 1892 (±421) | 3.2 (±1.7) |
| DCDV | Amp. | 4827 (±1833) | 0.25 (± 0) | 0.69 (± 0.67) | 2.28 (±2.7) | 4939 (±1343) | 4941 (±1344) | 0.73 (±0.2) |
| DCDV | Sul. | 4456 (±1337) | 0.25 (± 0) | 1.15 (±0.45) | 0.69 (±0.28) | 4034 (±888) | 4034 (± 888) | 0.6 (±0.12) |
| Jug. Vein | Amp. | 2.54 (±1.57) | 5.4 (±4.5) | | | | | |
| Jug. Vein | Sul. | 1.44 (±0.51) | 1.58 (±0.20) | | | | | |
| APPV | Amp. | 5422‡ (±1953) | | | | | | |
| APPV | Sul. | 5261‡ (±2038) | | | | | | |

$C_{max}$= maximum concentration; $T_{max}$= time at maximum concentration; $\lambda_z$= decay constant; $AUC_{0-t}$= area under the curve to final time point; $AUC_{0-inf}$= area under the curve extrapolated to infinity; $MRT_{0-inf}$= mean resonance time extrapolated to infinity; Syn= synovial; Amp= ampicillin; Sul= sulbactam; DCDV = dorsal common digital vein; APPV = abaxial proper plantar vein, Jug= jugular.

‡ Single time point concentration at 0.25 hours

Table 3: Limits of Quantification

| **Sample** | **Drug** | **Lower Limit of Quantification** |
|---|---|---|
| Synovial Fluid | Ampicillin | 5 µg/mL |
| Synovial Fluid | Sulbactam | 5 µg/mL |
| Serum | Ampicillin | 0.05 µg/mL (50ng/mL) |
| Serum | Sulbactam | 0.1µg/mL (100ng/mL) |

Figure 7: Mean Concentrations of Ampicillin in Synovial Fluid

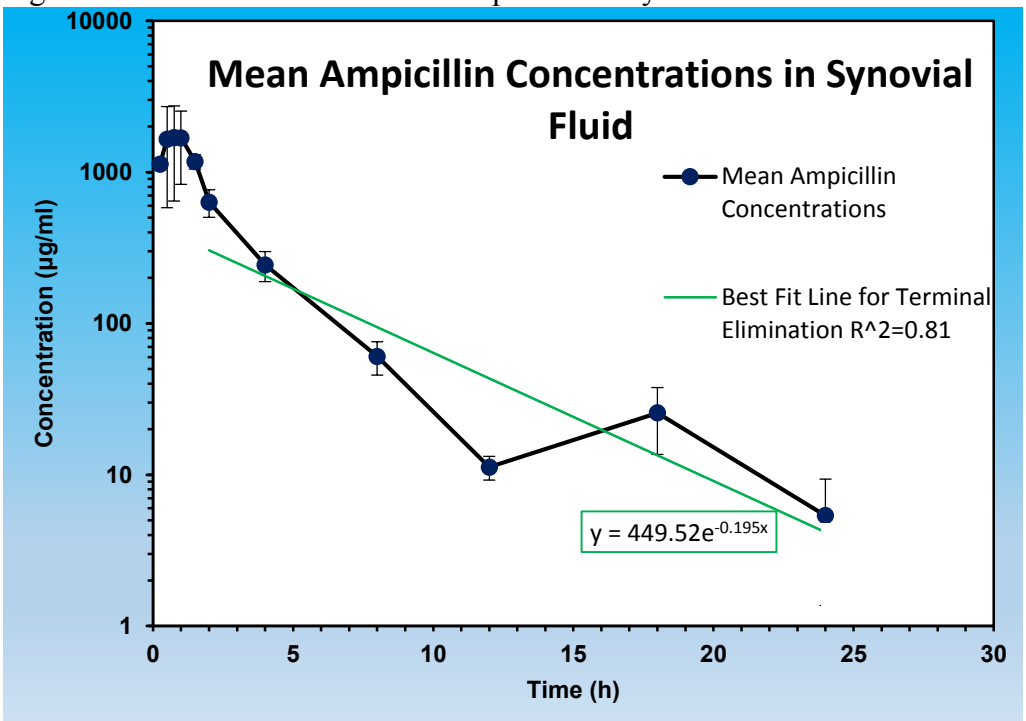



Table 4: Summary of T> MICs of Ampicillin in Synovial Fluid

| Animal # | T> 0.25 µg/mL (hours) | T>0.5 µg/mL (hours) | T> 1 µg/mL (hours) | T> 2 µg/mL (hours) | T>4 µg/mL (hours) | T>8 µg/mL (hours) | T>16 µg/mL (hours) | T>32 µg/mL (hours) |
|---|---|---|---|---|---|---|---|---|
| 1 | 37.4 | 34.3 | 31.2 | 28.0 | 24.9 | 21.7 | 18.6 | 15.5 |
| 2 | 41.9 | 38.4 | 34.8 | 31.2 | 27.7 | 24.1 | 20.6 | 17.0 |
| 3 | 25.9 | 23.8 | 21.7 | 19.6 | 17.5 | 15.4 | 13.3 | 11.2 |
| 4 | 33.4 | 29.8 | 26.2 | 22.6 | 19.0 | 15.3 | 11.7 | 8.1 |
| 5 | 37.2 | 34.1 | 31.0 | 27.9 | 24.8 | 21.6 | 18.5 | 15.4 |
| 6 | 25.4 | 23.4 | 21.4 | 19.3 | 17.3 | 15.2 | 13.2 | 11.2 |
| **Mean** | **33.5** | **30.6** | **27.7** | **24.8** | **21.8** | **18.9** | **16.0** | **13.1** |
| **St. Dev** | **6.7** | **6.08** | **5.5** | **5.0** | **4.5** | **4.04** | **3.7** | **3.4** |

Figure 8: Terminal Ampicillin Elimination in Synovial Fluid Extrapolated to 40 Hours with a Range of MICs

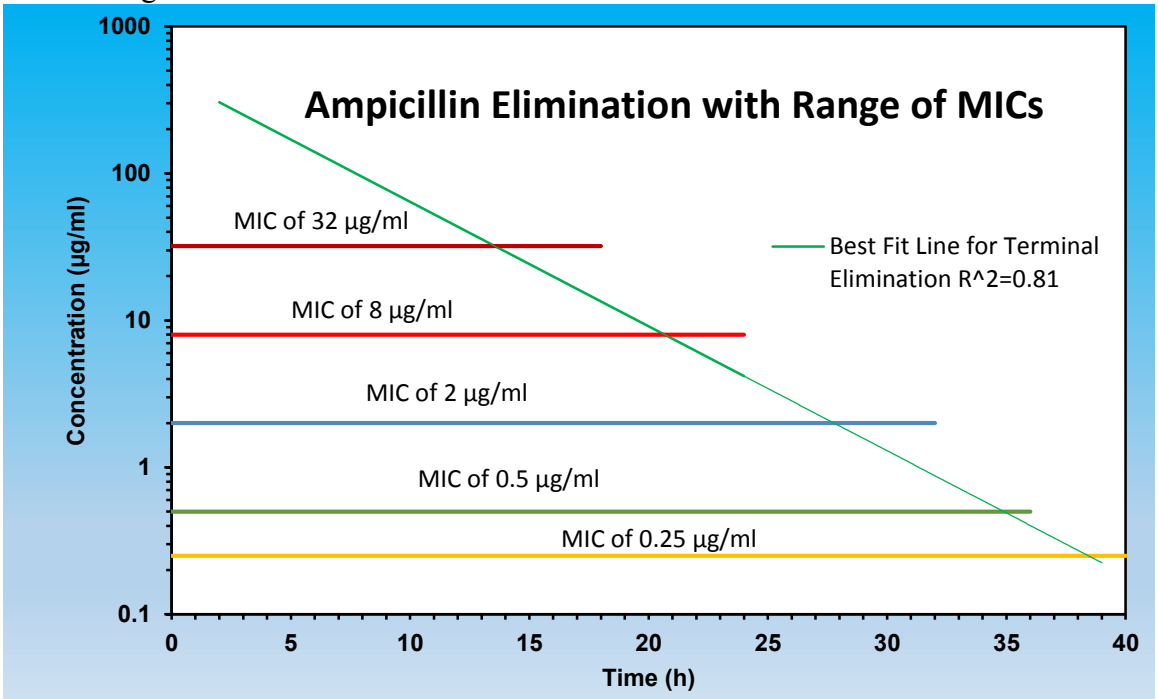


Figure 9: Ampicillin and Sulbactam Concentrations in Synovial Fluid (± St. Dev)

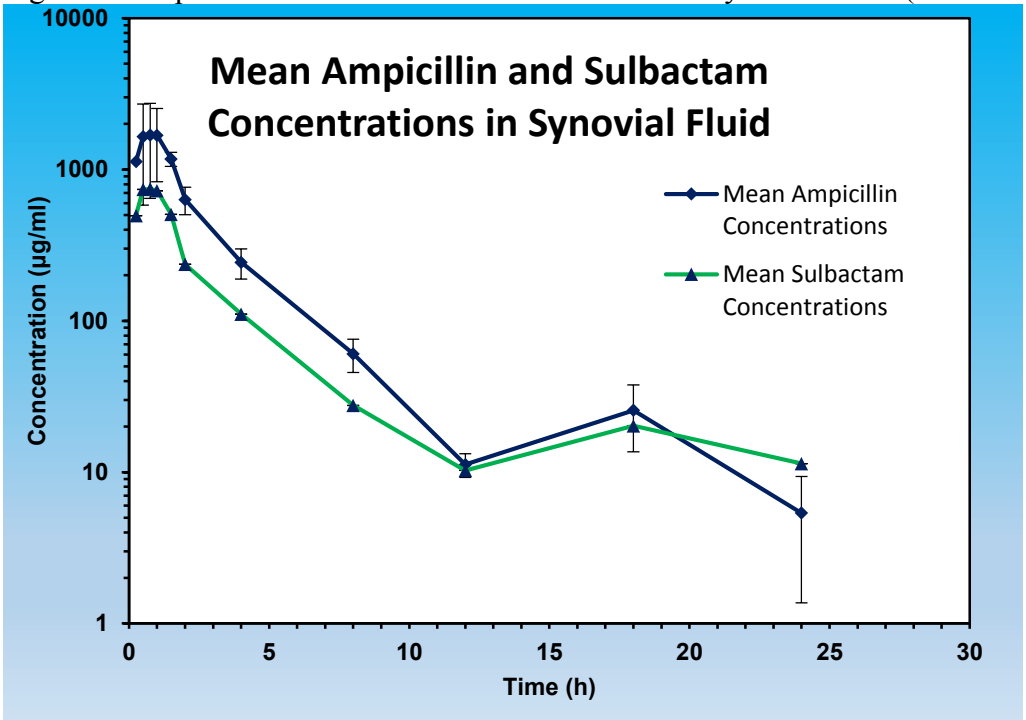
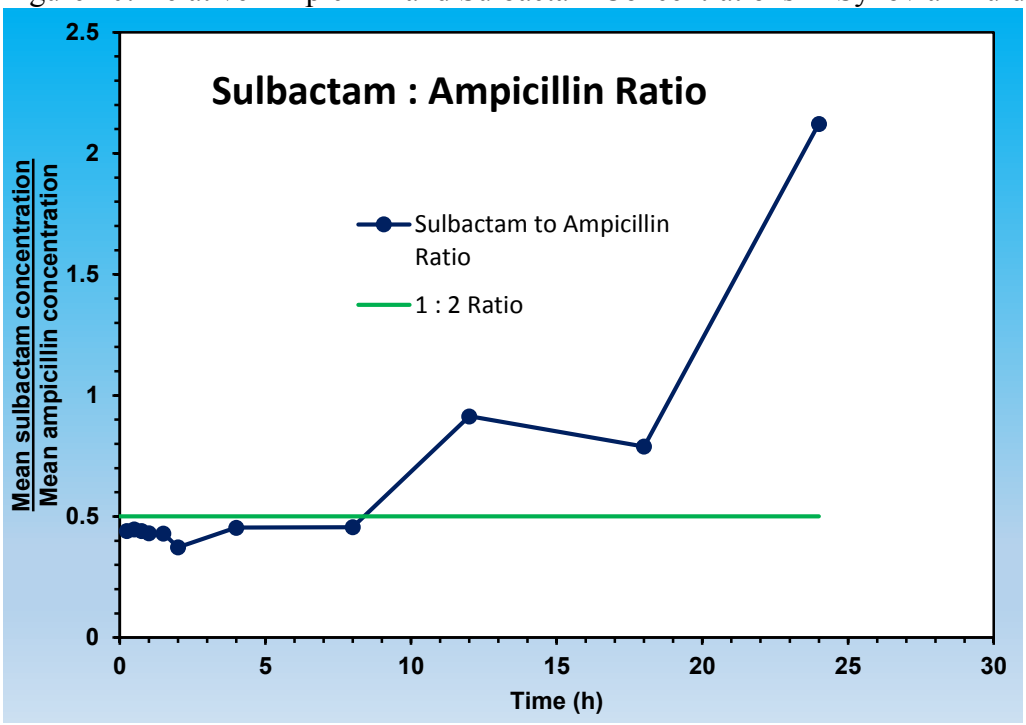



Figure 10: Relative Ampicillin and Sulbactam Concentrations in Synovial Fluid



# Chapter 5: Discussion

## 5.1 Interpretation of results

The high concentrations of both ampicillin and sulbactam achieved in digital circulation and synovial fluid demonstrate the diffusion of both drugs from the site of administration within the area distal to tourniquet application. Ampicillin is a time dependent antimicrobial, and as such it is important to maintain therapeutic concentrations for at least approximately half the dosing interval[11,47]. Ampicillin concentrations in synovial fluid remained above 8μg/mL, the CLSI breakpoint for human isolates, for approximately 19 hours, and above equine and porcine breakpoints for greater than 30 hours (Table 4). This suggests a significantly longer potential duration of therapeutic concentrations in synovial fluid after RLP than would have been estimated by examining the pharmacokinetics following systemic administration and elimination[72,74,75]. A range of MIC values were used to compare to the best fit line for the terminal elimination of ampicillin in synovial fluid. The resulting mean T> MIC is the range of time the drug can be expected to be greater than this MIC. This value should be at least half of the dosing interval for time dependent antibiotics. This can be used clinically by comparing a known MIC for culture and susceptibility to plan treatment intervals. Additionally, before such results are available, a range of likely MICs can be presumed

based on the most likely organisms present and the likely range of T>MIC can be predicted to estimate the dosing interval. For example, when planning a 24 hour dosing interval, the range of T>MIC must be greater than 12hours. With the data presented in Table 4, it can be recommended that any bacteria with an MIC of approximately 16 μg/mL or below, could be treated with a once daily RLP.

Drug concentrations at 18 hours showed an increase in both ampicillin and sulbactam in synovial fluid compared to the previous sampling at 12 hours, as illustrated in Figure 9. The cause for this is unknown, but could be speculated to result from diffusion from the tissues into the synovial fluid, or potentially due to contribution from systemic drug concentrations present after tourniquet release pumping back to the distal extremity; neither of which were specifically investigated in this study. The presence of this second peak may lead to an overestimation of AUC values, which could also cause the relatively low MRT for the given $T_{1/2}$ of some animals (animal #1, 4; Appendices 1,2). Another reason for decrease in MRT relative to $T_{1/2}$ is iatrogenic elimination of drugs from the DCDV during sampling, and thus drug elimination during the distribution phase.

Another important finding is that sulbactam concentrations followed similar trends to ampicillin as illustrated in Figure 9 and 10. This allows for adequate concentrations of sulbactam relative to ampicillin to be present in synovial fluid, which may be important for efficacy of both drugs[10]. Interestingly, in the latter time points, sulbactam levels were greater than half that of ampicillin levels. This may represent the slower renal elimination of sulbactam described in other ruminant studies[72,75,76]. This

relative increase in sulbactam is unlikely to affect drug efficacy, as only an insufficient amount of sulbactam relative to ampicillin is of potential concern to create a lack of sufficient β-lactamase inhibition for a given concentration of ampicillin.

The jugular venous samples, which represent systemic exposure to the drug, show significantly lower peak drug concentrations than that of synovial fluid and DCDV samples. Although there was insufficient data to reliably calculate other PK parameters from jugular venous samples, the significantly lower $C_{max}$ of both drugs as well as the inability to detect any drug in later time points (Table 2), (Appendices 5,6) suggests that systemic exposure is significantly less than regional drug exposure.

The $C_{max}$ and $T_{max}$ (Table 2) of both drugs were significantly lower in jugular venous samples than any of the digital sites, which indicated that both drugs reach systemic circulation at much lower levels than the distal extremity perfused and that these concentrations peak after release of the tourniquet. Three of six cattle had no detectable ampicillin in systemic circulation by 24 hours, one animal had concentrations below the LOQ and the other two were near the LOQ. Sulbactam concentrations were likewise low in systemic circulation; in two of six animals drug was undetectable by 8 hours and below the LOQ in one animal by 8 hours; in the remaining 3 animals the mean concentration at 24 hours was near the LOQ. This information suggests that a significant portion both drugs has been eliminated by 24 hours. Although local drug concentrations in the circulation and synovial fluid of the distal extremity were significantly higher, this part of the carcass (from the hock or carpus to the foot) is trimmed out at processing and would not enter the human food supply. Additionally, ampicillin undergoes primarily

renal excretion; thus to obtain a better estimate of true drug clearance, urine drug concentrations could be measured over time. Tissue samples and particularly renal tissue could be analyzed to confirm accurate withdrawal recommendations after RLP. Until such information is available, the more conservative estimate of a meat withdrawal time of 6 days suggested by FARAD is prudent.

5.2 Study limitations and confounders

Initially, the APPV was intended to be the sampling site for all digital venous blood samples. However, this site proved to be unreliable for patent venous catheter placement. The dorsal common digital vein was then used as the alternative digital venous sampling site, with the exception of a single venous sample from the abaxial plantar vein of the lateral digit taken via routine phlebotomy as described above. This was not ideal, as sampling blood from the same catheter used for drug administration can introduce error from drug residue in the catheter. This was minimized by catheter flushing after drug administration and between each sample. One likely consequence of this sampling technique is artificially high drug concentrations measured from samples from the DCDV. However, the presence of high regional drug concentration is likely an accurate finding, as drug concentrations measured from the APPV were very high, higher even than those measured from the DCDV at the same time point (Table 2), (Appendices 3,4,7,8). Additionally, synovial fluid drug concentrations were of a similar order of magnitude to those measured from the DCDV (Table 2) (Figure 6). Another consequence of this sampling technique is removal of drug directly from the local

circulation before tourniquet release; this leads to sampling induced drug elimination during the time the drug is still being distributed through the limb. This elimination might explain why the MRT is less than the $T_{1/2}$ (Table 2).

A pneumatic tourniquet was originally intended to be used for occlusion of digital blood flow during regional limb perfusion as described by Gilliam et al 2008[31]. Due to unreliable calibration of the device, the decision was made to use the manual tourniquet technique described. Additionally, this type of tourniquet is a type commonly utilized in clinical practice. Based on the study by Alkabes et al in 2011, the wide rubber tourniquet used in this study should be adequate for the purpose of RLP[53]. During the first perfusion, the second layer (rubber tubing) was not applied to tourniquet. The tourniquet remained in place for this animal, however rubbing of the tourniquet on the chute was noted, so a second tubing layer was added for all of the following perfusions to prevent damage to the tourniquet.

Low or undetectable drug concentrations in systemic circulation (Table 2), (Appendices 5,6) lead to insufficient data points above the LOQ to allow for significant PK analysis of either drug in jugular venous samples. If a more sensitive method of detection were chosen, drug concentration could be quantified and AUC calculations could be used to quantitatively compare systemic exposure to local exposure. However, these data still demonstrate that systemic concentrations are significantly lower than local drug concentrations. Another potential advantage of obtaining a PK analysis of both drugs from jugular venous samples would be for use in calculation of a withdrawal period. A standard calculation of ten times the $T_{1/2}$ estimates the time at which 99.9% of

the drug should be eliminated[91]. In the absence of these calculations, the low $C_{max}$ of both drugs on jugular venous samples, as well as the absence of detectable drug levels in many of the later time points (Appendices 5,6) suggests that much of the drug was eliminated by 24 hours. Using the meat withdrawal time of 6 days suggested by FARAD, it is unlikely that violative drug residues will be present after use of 1.5g combined ampicillin-sulbactam in adult cattle as an RLP.

An unexpected result was that some non-zero drug concentrations were detected in some DCDV and jugular venous samples prior to regional limb perfusion. This was surprising because all study animals were housed on university property for at least 6 months prior to initiation of the study, and none had been treated with any pharmaceuticals containing ampicillin or sulbactam within this time. It was suspected that these levels were a result of sample processing, and were due to HPLC column contamination from extremely high concentrations of drug in samples analyzed before the time zero samples were analyzed. Interestingly, Gagnon et al described a similar problem during the study of cefazolin when used as an RLP in cattle[28].

The cattle used in this study were not lactating during the study period. Although this limited any variability that might be introduced by the high metabolic demand of lactation as well as potential differences in milk production, it may have been useful to obtain milk samples during the study to estimate potential milk withdrawal times for ampicillin-sulbactam after administration as an RLP. Using the data presented here, and a known milk to plasma ratio for ampicillin of 0.3:1, peak milk ampicillin concentrations

may approximate 0.85 μg/mL, and this peak may occur at approximately 5 hours post RLP[92].

# Chapter 6: Conclusions and Clinical Relevance

Rational use of antimicrobials is an increasingly important component of food animal medicine. This study documents the concentration vs time curve of ampicillin-sulbactam when used as an RLP in the distal hind limb of healthy cattle. When choosing an antimicrobial for use as an RLP in the treatment of DDS, many considerations must be taken into account. The drug must reach therapeutic concentrations at the site of infection, the deeper tissues of the bovine distal extremity. The data reported herein demonstrate that high concentrations, well above therapeutic concentrations, are reached in the area distal to tourniquet application, and are present in both vascular and synovial fluid in the distal extremity.

For time dependent antimicrobials, including β-lactams, therapeutic concentrations must persist for greater than half of the dosing interval for maximum effectiveness. Based on the results presented here, ampicillin-sulbactam could be administered at a convenient once daily dosing interval for a wide range of MICs. The drug utilized should be safe when given IV. In this study, the procedure was well tolerated, and no adverse events were encountered.

Although not specifically investigated in this study, the properties of ampicillin-sulbactam[z] may also allow for the simultaneous administration of 2% lidocaine during an RLP and thus aid in the clinical treatment of DDS.

And, of great importance, administration of the drug should be legal under AMDUCA and not create any violative residues. Ampicillin-sulbactam is an FDA approved drug, labeled for use in humans for treatment of bacterial infection that include gram positive aerobic bacteria, anaerobic bacteria and coliforms, specifically including both those susceptible to ampicillin as well as β-lactamase producing bacteria. . Although it is not labeled for use in cattle, no products are labeled for use as RLPs in any food animal, thus necessitating consideration of off label drug use for any use of RLPs in cattle. Off label use of ampicillin-sulbactam is not prohibited by the FDA providing the drug is prescribed by a veterinarian, and tissue tolerances have been established for ampicillin in meat and milk. Withdrawal times and tissue tolerances do not exist for sulbactam, however it has a similar half-life to ampicillin and little risk for toxicity[86]. Following a single dose of 1.5 grams combined ampicillin (1g) and sulbactam (0.5g) administered as an RLP in a 1000 pound animal, meat withdrawal has been suggested by FARAD to be 6 days, and milk withdrawal to be 48 hours[87]. Based on the low systemic drug concentrations described in this study, the use of ampicillin-sulbactam as an RLP does not appear likely to result in residues above tissue tolerances after adhering to the withhold recommendations described by FARAD.

Further research evaluating the efficacy of ampicillin-sulbactam for treating clinical cases of DDS when used as an RLP, as well as the use of these drugs in combination with regional anesthesia are warranted. Based on the very high concentrations reached in this study, assessing pharmacokinetic data or efficacy with a

lower dose may allow for rational usage of an even lower dose of ampicillin-sulbactam as an RLP.

The results indicate that the use of 1.5g combined ampicillin and sulbactam is a relatively safe procedure that achieves therapeutic concentrations for a significant portion of a 24 hour dosing interval for the potential treatment of DDS in cattle. These data suggest that ampicillin-sulbactam is potentially clinically useful in cases of DDS due to susceptible bacteria, and would be a rational and economically feasible antimicrobial choice when given as a once daily RLP for therapy of deep digital sepsis in cattle.

Endnotes:

a. Xylazine HCl 20mg/ml: Lloyd laboratories, Shenandoah IA, 51601
b. Chlorohex: ChlorHex-Q Scrub, VEDCO, St. Joseph, MO 64507
c. Alchohol: Isopropyl rubbing alcohol 70%, HUMCO, Texarkana, Tx 75501
d. Lidocaine 2%: VetOne, Boise ID, 83705
e. Jugular catheters: Milacath extended use, 14g x 13cm catheter, Mila international incorporated, Erlanger KY, 41018
f. Extension sets: braun extension set, 18cm 1ml priming volume, B Braun medicals incorporated, Bethlehem PA, 18018
g. Injection ports: Terumo, Elkton, MD, 21921
h. Braunamid: 1 Braunamid. B Braun medicals incorporated, Bethlehem PA, 18018
i. 18g x 1.5" needle: Covidien, Mansfield MA, 02048.
j. Heparinized saline: 10,000 units of heparin[u] was added 1L normal 0.9% saline[m]
k. Betadyne scrub: Poviderm Medical Scrub, VetUS, Dublin OH 43017
l. Tourniquet: Esmark Bandage, 4" x 9'. Owens and Minor, Machanicsville, VA 23116.
m. Saline:0.9% Sodium Chloride 1L, Baxter, Deerfield, IL 60015
n. Epidural pain management kit: 20g catheter, 18gx7.5cm Touhy needle. Mila international, Erlanger KY, 41018
o. 16g x 1" needle: Covidien, Mansfield MA, 02048.
p. Surgipro 2-0 monofilament polypropylene suture: Covidien, Mansfield MA, 02048.
q. Skin staples: Covidien, Mansfield MA, 02048.
r. Guidwire: 0.18 x 45cm, Mila, Erlanger, KY 41018
s. 18g x 2"catheter in DCDV: Surflo IV catheter, Teruma medical corp. Somerset, NJ 08873
t. Superglue: Duro, Pacer Technologies, Rancho Cucamonga, CA 91730
u. Heparine: Heparin Sodium, 10,000 USP/10ml, NOVAPLUS, Schaumburg, IL 60173
v. Rolled gauze: Curity stretch bandage.6" x 82". Covidien, Mansfield MA, 02048.
w. PowerFlex: 4" x 5 yards. Andover. Salisbury, MA01952
x. Duct tape: Shurtape. Avon, OH 44011
y. Elastikon: 3" x 2.5 yards. Johnson and Johnson. Skillman NJ 08558-9418
z. Ampicillin Sulbactam 1.5g vial: 1g ampicillin, 0.5g sulbactam. Aeromedics Pharma, Dayton, NJ 08810
aa. Butterfly needles: SURFLO Winged Infusion Set 19Gx3/4", TERUMO corperation Tokyo 151-0072, Japan
bb. Red top tubes: sterile glass blood collection tubes, Covidien, Mansfield MA, 02048.

cc. Epindorph tubes 2ml: Eppendorf manufacturing, North America
dd. Freezer -80: So low, Cincinnati, OH 45215
ee. Ampicillin Na for standard: Ampicillin for injection, 1g vial, Sandoz GmbH, Princeton, NJ 08540
ff. Sulbactam analytical reference material: 10mg. Sigma Aldrich, St. Louis, MO 63103
gg. Solid phase extraction column: Oasis HLB, Waters Millipore
hh. Reverse phase column: Agilent Technologies, Wilmington, DE, USA

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## Appendix: Original Data

Sulbactam Concentrations in Synovial Fluid Samples

| Synovial Fluid Samples (samples treated with hyaluronidase before processing) | | | | | | |
|---|---|---|---|---|---|---|
| **SULBACTAM** | | | | | | |
| **Animal Number** | | | | | | |
| **Time (Hr)** | **1** | **2** | **3** | **4** | **5** | **6** |
| **0** | nd | nd | nd | nd | nd | nd |
| **0.25** | 857.5136 | 399.9 | 583.4716 | 722.6106 | 301.4133 | 114.1507 |
| **0.5** | 964.1726 | 510.0 | 802.533 | 1337.831 | 639.7893 | 171.6042 |
| **0.75** | 1136.769 | 505.8 | 671.7681 | 1223.171 | 634.2884 | 299.9578 |
| **1** | 1166.289 | 682.2 | 879.8324 | 479.8079 | 775.4774 | 369.6355 |
| **1.5** | 498.0445 | 628.3 | 737.4726 | 205.395 | 493.7107 | 468.6512 |
| **2** | 157.0254 | 444.0 | ns | 19.87639 | 325.6286 | 233.9474 |
| **4** | 42.4472 | 134.3 | 111.3937 | 42.10495 | 145.1546 | 188.1836 |
| **8** | 56.05911 | 26.8 | 11.2 | 6.952316 | 20.3154 | 44.36642 |
| **12** | 15.37009 | 5.2 | 3.2 | 3.699299 | ns | nd |
| **18** | 24.19977 | 37.3 | 3.7 | 1.856925 | 19.49027 | nd |
| **24** | 13.61294 | 9.2 | <loq | ns | 0.176104 | nd |

Ampicillin Concentrations in Synovial Fluid Samples

| Synovial Fluid Samples (samples treated with hyaluronidase before processing) | | | | | | |
|---|---|---|---|---|---|---|
| **AMPICILLIN** | | | | | | |
| **Animal Number** | | | | | | |
| **Time (Hr)** | **1** | **2** | **3** | **4** | **5** | **6** |
| **0** | nd | nd | nd | nd | nd | nd |
| **0.25** | 2304.515 | 854.6 | 1049.062 | 1647.001 | 534.9797 | 367.2358 |
| **0.5** | 2671.605 | 989.4 | 1492.889 | 3196.142 | 1119.294 | 419.919 |
| **0.75** | 3235 | 1030.2 | 1300.203 | 2773.047 | 1147.361 | 667.2307 |
| **1** | 3319.059 | 1332.2 | 1674.701 | 1426.621 | 1522.814 | 817.4583 |
| **1.5** | 1569.227 | 1303.0 | 1409.63 | 785.3527 | 1052.577 | 922.5945 |
| **2** | 523.2741 | 1025.7 | ns | 229.7226 | 754.8826 | 632.9522 |
| **4** | 93.31816 | 369.0 | 248.9262 | 76.95743 | 279.8336 | 392.8723 |
| **8** | 115.2651 | 57.0 | 32.84476 | 27.42439 | 38.51482 | 92.39428 |
| **12** | 17.81735 | 10.0 | 10.0 | 16.4987 | ns | 1.855166 |
| **18** | 36.00073 | 70.1 | 9.4 | 2.890425 | 34.53483 | 1.026959 |
| **24** | 7.523929 | 15.9 | 0.4 | | 2.453316 | 0.58985 |

Sulbactam Concentrations in Dorsal Common Digital Vein Samples

| SULBACTAM | | | | | | |
|---|---|---|---|---|---|---|
| **Animal Number** | | | | | | |
| **Time (Hr)** | **1** | **1** | **3** | **4** | **5** | **6** |
| **0** | nd | nd | nd | nd | nd | nd |
| **0.25** | 4061.79 | 4269.476 | 2557.503 | 3893.116 | 5673.502 | 6283.129 |
| **0.5** | 2818.347 | 2567.351 | 1508.175 | 2219.584 | 3062.88 | 2284.695 |
| **0.75** | 2228.561 | 1957.638 | 1235.691 | 1995.579 | 1771.665 | 1680.926 |
| **1** | 1632.477 | ns | 1144.788 | 1437.671 | 1313.842 | 1132.363 |
| **1.5** | ns | ns | ns | 225.6941 | ns | 266.3757 |
| **2** | ns | 234.4637 | 149.278 | ns | ns | 120.8544 |
| **4** | 5.026738 | 22.9808 | 19.44583 | ns | 19.83792 | 2.629756 |
| **8** | 0.13076 | 1.424898 | 0.86457 | 0.843055 | 0.638777 | ns |
| **12** | 0 | ns | 0.168938 | nd | ns | nd |
| **18** | <loq | ns | 0.161767 | nd | nd | nd |
| **24** | 0.056745 | 1.119174 | 0.503128 | nd | nd | nd |

Ampicillin Concentrations in Dorsal Common Digital Vein Samples

| AMPICILLIN | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Animal Number** | | | | | | | | |
| **Time (Hr)** | **1** | **2** | **3** | **4** | **5** | **6** | **Mean** | **SD** |
| **0** | 2.300055 | 0 | 0 | 0.115355 | 0 | 0 | 0.402568 | 0.930719 |
| **0.25** | 3057.43 | 8044.011 | 3373.363 | 4928.888 | 5552.02 | 4004.302 | 4826.669 | 1833.383 |
| **0.5** | 2331.097 | 4450.439 | 1719.004 | 3093.706 | 4602.147 | 2931.521 | 3187.986 | 1145.467 |
| **0.75** | 1969.017 | 3372.728 | 1806.872 | 3460.389 | 4110.514 | 3647.854 | 3061.229 | 945.2858 |
| **1** | 1634.093 | ns | 1743.697 | 2895.809 | 2746.558 | 1928.87 | 2189.805 | 588.2883 |
| **1.5** | 189.9364 | 596.9129 | 1574.807 | 448.8067 | 482.0301 | 391.82 | 614.0522 | 489.3548 |
| **2** | 38.88018 | 221.7606 | 209.9163 | 169.6242 | 347.7903 | 173.245 | 193.5361 | 99.76095 |
| **4** | 11.89676 | 3.924511 | 190.4558 | ns | 57.09356 | 1.058179 | 52.88576 | 80.16643 |
| **8** | 1.135364 | nd | 1.735459 | 4.459743 | 2.540021 | 3.31303 | 2.197269 | 1.590603 |
| **12** | 0.145075 | ns | 0.215734 | 2.858581 | 1.720632 | 0.251916 | 1.038388 | 1.211597 |
| **18** | 0.026192 | ns | 0.120269 | 1.370444 | 0.205536 | 0.06794 | 0.358076 | 0.569876 |
| **24** | <loq | nd | nd | 1.103703 | 0.174616 | 0.089585 | 0.273581 | 0.469692 |

Sulbactam Concentrations in Jugular Venous Samples

| SULBACTAM | | | | | | |
|---|---|---|---|---|---|---|
| **Animal Number** | | | | | | |
| **Time (Hr)** | **1** | **2** | **3** | **4** | **5** | **6** |
| **0** | nd | 1.114518 | nd | nd | nd | nd |
| **0.25** | 0.637838 | 1.106758 | 0.712535 | 0.153161 | nd | nd |
| **0.5** | 0.861456 | 0.749821 | 0.754129 | 0.085749 | nd | nd |
| **0.75** | 0.645712 | 0.580664 | 0.471574 | <loq | nd | nd |
| **1** | 1.089798 | 0.413059 | 0.257864 | <loq | 0.123413 | nd |
| **1.5** | 2.444106 | 1.176594 | 1.388086 | 1.1586 | 1.099815 | 1.239301 |
| **2** | 2.27088 | 1.057098 | 1.531516 | 0.696758 | 0.455243 | 0.819374 |
| **4** | 0.165405 | 0.971743 | 0.734049 | 0.090052 | ns | 0.292264 |
| **8** | 0.215798 | 0.260972 | <loq | nd | 0.466989 | nd |
| **12** | 0.124461 | 0.105782 | <loq | nd | 0.111667 | nd |
| **18** | 0.135484 | 0.053018 | <loq | nd | 0.220319 | nd |
| **24** | 0.433117 | 0.409955 | 0.210533 | nd | 0.453774 | nd |

Ampicillin Concentrations in Jugular Venous Samples

| AMPICILLIN | | | | | | |
|---|---|---|---|---|---|---|
| **Animal Number** | | | | | | |
| **Time (Hr)** | **1** | **2** | **3** | **4** | **5** | **6** |
| **0** | nd | nd | nd | 0.084469 | 0.358592 | nd |
| **0.25** | 0.117919 | nd | 0.094297 | 0.560392 | 0.112002 | 0.057891 |
| **0.5** | 0.029209 | nd | 0.225562 | 0.296459 | 0.067167 | nd |
| **0.75** | 0.015933 | nd | 0.113951 | 0.240303 | 0.07026 | nd |
| **1** | 0.035244 | nd | 0.326643 | 0.200291 | 0.658519 | 0.066394 |
| **1.5** | 1.527617 | nd | 1.335347 | 1.152839 | 1.635602 | 1.295478 |
| **2** | 0.75518 | nd | 1.012449 | 0.942254 | 1.249097 | 1.094496 |
| **4** | 0.068434 | nd | 1.651225 | 0.219244 | 2.246278 | 0.309119 |
| **8** | nd | nd | 0.19257 | 0.216436 | 3.599043 | 0.224088 |
| **12** | nd | nd | 0.222052 | 0.108336 | 2.40088 | 4.766286 |
| **18** | nd | nd | 0.191166 | 0.296459 | 3.552662 | 3.34395 |
| **24** | nd | nd | 0.114653 | nd | 0.078763 | 0.04475 |

Sulbactam Concentrations in Abaxial Proper Plantar Venous Samples

| | SULBACTAM | |
|---|---|---|
| **Animal Number** | **Time** | **µg/ml** |
| **1** | 15m | ns |
| **2** | 15m | 4276.589 |
| **3** | 15m | 2924.551 |
| **4** | 15m | 4322.007 |
| **5** | 15m | 7051.913 |
| **6** | 15m | 7728.752 |

Ampicillin Concentrations in Abaxial Proper Plantar Venous Samples

| | AMPICILLIN | |
|---|---|---|
| **Animal Number** | **Time** | **µg/ml** |
| **1** | 15m | 3327.52 |
| **2** | 15m | 2928.956 |
| **3** | 15m | 5663.333 |
| **4** | 15m | 5687.914 |
| **5** | 15m | 7429.039 |
| **6** | 15m | 7499.537 |

SD= standard deviation of the mean; SE= standard error of the mean; nd= not detected; ns= not sufficient sample volume for processing; <loq= value below the limit of quantification.
*Values below the limit of quantification were removed from data analysis.*
*Highlighted values were classified as outliers and removed from data analysis*