Summer 8-2014

EVALUATION OF PRE-HARVEST SCREENING FOR ANTIBIOTICS AND FLUNIXIN MEGLUMINE IN CATTLE

Shuna A. Jones
University of Nebraska-Lincoln, shuna.jones@ars.usda.gov

EVALUATION OF PRE-HARVEST SCREENING FOR ANTIBIOTICS AND FLUNIXIN MEGLUMINE IN CATTLE

by

Shuna A. Jones

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Veterinary Science

Under the Supervision of Professor Dee Griffin

Lincoln, Nebraska

August, 2014

EVALUATION OF PRE-HARVEST SCREENING FOR ANTIBIOTICS

AND FLUNIXIN MEGLUMINE IN CATTLE

Shuna A. Jones, M.S.

University of Nebraska, 2014

Advisor: Dee Griffin

Many antibiotics and medications used in the treatment of animals have a withdrawal time; residues are a concern for the meat industry. The most recently published 2009 USDA-FSIS Residue Program Data Report listed 135,389 Inspector-In-Charge-Generated (IICG) residue samples from 43,142,500 beef and dairy cattle inspected that year at harvest (USDA-FSIS 2009a). Of these samples, 1306 contained violative antibiotic residues and 327 violative flunixin meglumine residues. Two classes of antimicrobials comprised over half of the documented violative antimicrobial residues (beta-lactams that include ceftiofur and sulfa drugs that include sulfadimethoxine). While the violative residue rate seems small, violative residues are unacceptable. Management of carcasses that contain violative residues is costly to the USDA, the meat packing industry, and the producers involved.

There is no preharvest or ante-mortem screening test currently available that mirrors the antibiotic screening test used by the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) in beef and swine packing plants (USDA-FSIS Notice 39-09). Adapting the test currently used by the USDA-FSIS to screen for antibiotic residues in tissue or used by the Food and Drug Administration

(FDA) to screen for drug residues in milk would make it possible for producers and veterinarians to identify or predict livestock that might be considered a high risk for containing a violative antibiotic residue in tissue. In addition, there is no preharvest screening test currently available to detect violative residue levels of flunixin meglumine and ceftiofur in cattle prior to market (Damian, 1997).

A simple, cow-side test for the presence of drug residues in live animal fluids would provide useful information for tissue drug residue avoidance programs. This work describes adaptation and evaluation of rapid screening tests to detect drug residues in serum and urine. Medicated herd animals had urine, serum, and tissue biopsy samples taken during drug treatment. Samples were tested by rapid methods and high-performance liquid chromatography (HPLC). The adapted microbial inhibition method, kidney inhibition swab (KIS®) test, was useful in detecting sulfadimethoxine in serum and successfully predicted proper drug withdrawal in the kidney by HPLC, 5 to 6 days post treatment. The lateral flow (LF) screening method for flunixin and beta-lactams adapted for urine was useful in predicting flunixin in the liver detected by HPLC, 96 hours post-treatment. The same adapted methods were not useful to detect ceftiofur in serum or urine relative to the recommended tissue withdrawal time after ceftiofur treatment. These anti-mortem screening tests demonstrated that the selection of method used, and whether urine or serum are tested, will vary based on drug used and should be based on animal treatment history if available. The live animal tests demonstrated the potential to allow verification that an individual animal is free of drug residues before sale for human consumption.

Table of Contents

Chapter 1

Introduction

The use of antimicrobials and other approved medications in animal production for treatment, prevention, or nutritional purposes inevitably results in the presence of residues in animal tissue. To protect consumers from exposure to potentially harmful compounds or residue levels, the use of these pharmaceuticals demands that simple and reliable methods are available to screen for these compounds prior to entry in the food chain. A growing concern among consumers and public health authorities on the use and presence of antimicrobial compounds in foods demands further improvement of quality management programs in animal production.

The March 2010, Food Safety Inspection Service (FSIS) National Residue Program (NRP) for Cattle Audit Report (24601-08-KC), concluded that the national residue program was not accomplishing their mission and identified several areas of concern that need to be rectified. The FSIS, Environmental Protection Agency (EPA), and Food and Drug Administration (FDA) were tasked to work together to find more efficient ways to test for residues, and set new acceptable residue levels.

Residues

Contaminated meat may contain residues from veterinary drugs, pesticides, and heavy metals. A drug residue is any substance that remains in the tissues of an animal

that has been treated with that substance. Surveillance programs detect drug residues in the small percentage of samples that are being tested. In 2008, FSIS found .003% positive residues in the samples that were collected from over 43 million cattle that were slaughtered (USDA-FSIS Red Book 2009a). The 135,389 is a biased sample because it's the animals that are railed off and checked by the FSIS veterinarians on the floor. The positives in this group would be over represented because animals that have some pathologic lesions and are more likely to have been treated with something. Focusing sampling on carcasses that are more likely to contain residues makes efficient use of limited resources; however, because of the biased sampling estimation of overall prevalence is not possible. The residues of veterinary drugs in meat, eggs and milk have lead to concerns about possible adverse effects they may have on consumers of these foods.

Residues are unlike microbiological pathogens such as *E. coli*, *Salmonella*, and *Listeria*, which the public readily associates with food safety. Cooking meat properly can destroy bacteria, but no amount of cooking will denature a residue completely (USDA-FSIS Audit Report 24601-08-KC). In some situations, heat may denature the residue into metabolites that are even more harmful to the consumer.

Veterinary Drugs

Since the discovery and development of antibiotics, veterinarians and producers have used antibiotics and other approved animal medications for the treatment and prevention of disease. It is to be expected that the greatest residue problems would be

connected to the most commonly used antibiotics and medications for treating cattle. Consistent with expectation, according to the USDA-FSIS (Audit Report 24601-08-KC) the top ten drugs that lead to illegal residues in cattle from 2005 to 2010 were: Penicillin, Flunixin, Sulfadimethoxine, Gentamicin, Ceftiofur, Sulfamethazine, Oxytetracycline, Neomycin, Tilmicosin, and Tetracycline.

Withdrawal Times

Approved medications by the U.S. Food and Drug Administration (FDA) are labeled for specific species at defined treatment dosages, and by approved routes of administration. The withdrawal time, which is printed on the label is defined as the time that is required for 99% of the animals in a population (treated according to label directions) to have drug residues that are lower than accepted residue levels defined by FDA. If any of the parameters that the recommended withdrawal time is based on (*i.e.* species, dosage, or route) change, then a new withdrawal time should be recommended to allow adequate drug metabolism and excretion by the patient and minimize unacceptable residues.

Under the Veterinary-Client-Patient relationship, veterinarians may choose to treat a patient with an approved product in a different way than is specified on the label; however, in doing so they must consider the effect on the withdrawal time and adjust it to match circumstances. Failure to follow the recommended withdrawal time period for approved animal drugs is the primary cause of violative levels in food (KuKanich, 2005). This failure often occurs due to inadequate record keeping, untrained labor, or

inappropriate dose or route of administration for the medication. If veterinarians are unsure what an appropriate withdrawal time should be, then the Food Animal Residue Avoidance and Depletion program (FARAD), located at http://farad.org/ which was developed by pharmacologists and toxicologists from the University of California-Davis, is available to veterinarians to provide educated guidance and make appropriate recommendations for withdrawal times for approved animal drugs used in different circumstances.

Effects of Residues on Human Health

Although not common, drug residues in meat have been reported to cause adverse health effects in people who have toxic or allergic reactions to the specific drug or drug metabolites after consumption of the product (Martinez, 2005). The most memorable event occurred in December of 2005, when 225 people consumed Mexican beef that had residues of clenbuterol, a growth promoter that is now illegal for use. Another human illness outbreak also associated with clenbuterol occurred in 1990 in Spain where 125 people consumed meat from animals treated with implants. In this later incident, some illnesses were attributed to the patients eating tissue that was near the implant location; in other cases, it was difficult to prove that hormone residues caused the illness.

If an individual is sensitive to specific medications or metabolites of a medication, they may experience an allergic reaction to antibiotic residues. It is estimated that about 7% of the general population have drug sensitivities to medications (Gomes, 2005). Penicillin residues in meat, in particular, have been documented as causing minor allergic

reactions when consumed by humans (Raison-Peyron, 2001). However, not all of the people with known drug sensitivities to medications experience symptoms when consuming meat with residues. This lack of response is likely because residue levels in meat are below threshold levels that would induce a hypersensitivity reaction: penicillin for example, is cleared rapidly from the blood through the kidneys into the urine. Results from the Joint Expert Committee on Food Additives (JECFA) in 1990 indicated that penicillin residues in the kidney and liver (as determined by HPLC) were about 100 times higher than those in muscle (Paturkar, 2005).

Justification

The latest USDA-FSIS Residue Report, which includes data following the FSIS changing residue screening procedures, listed 135,389 Inspector-In-Charge-Generated (IICG) residue samples from beef and dairy cattle (USDA-FSIS, 2009a [2008 FSIS National Residue Program Data]). Of these samples, 1,306 contained violative antibiotic residues and 327 violative flunixin meglumine residues. While all residues are of concern, two classes of antimicrobials, beta-lactams that include ceftiofur and sulfa drugs like sulfadimethoxine, comprise over half of the documented violative antimicrobial residues. The IICG samples were selected from the 43,142,500 bovine slaughtered under USDA inspection that year. The 135,389 is a biased sample because it's the animals that are railed off and checked by the FSIS veterinarians on the floor. The positives in this group would be over represented because they are the animals that have some pathologic lesions and are more likely to have been treated with something. Focusing sampling on

carcasses that are more likely to contain residues makes efficient use of limited resources; however, because of the biased sampling estimation of overall prevalence is not possible.

While the violative residue rate seems small, any violative residues are unacceptable as they are above the tolerances set by FDA and EPA. Management of carcasses that contain violative residues is costly to the USDA, the meat packing industry, and the producers involved. The costs are associated with losses due to carcasses being trimmed or condemned, and the additional work required for retaining and testing for residues. Most important may be the loss of consumer confidence in foods of animal origin, especially if carcasses with residues are not detected by screening programs.

Objectives

The Food and Drug Administration (FDA) has approved the use of the Kidney Inhibition Swab (KIS®) test for post-harvest screening for residues in animal tissues at slaughter. This project focused on the possibility of using the KIS® test for screening tissue and body fluids of the preharvest animal as an affordable option for evaluating residue status.

1. To determine if the Charm KIS® test technologies used by the USDA-FSIS to screen bovine kidneys for antibiotic residues will detect antibiotics in cattle urine.

2. To determine if the Charm Rapid One Step Assay (ROSA) test technologies, particularly the FLUBL Flunixin and Beta-lactam test, can be used to detect flunixin meglumine, sulfadimethoxine, and ceftiofur in cattle urine.

Chapter 2

Literature Review

All animal drugs have to be approved by FDA before they can be marketed for public use. Receiving FDA approval is a complicated and expensive process, as the drug developer must prove that the medication is safe and effective when used at the proposed labeled dosage. The New Animal Drug Application (NADA) must include all the possible side effects the drug may cause, and show that they can consistently manufacture the product with ingredients from safe and reliable sources. If the drug is for food animals, then withdrawal times (WDT) must be provided at the labeled dosage to insure that the residues in meat, milk, and eggs are below levels safe for human consumption http://www.fda.gov/.

Withdrawal Time

Before a drug can be used in a food-producing animal, experiments are conducted to provide evidence that the drug residues in animal tissues are below the approved maximum residue limit (MRL) after a certain amount of time. The FDA has proposed a regression method that would estimate that 99% of the population was below the approved MRL in the suggested withdrawal time with a 95% confidence level (Fisch et al., 2000). The withdrawal time (WDT) is the time needed after a treatment is completed for the tissue concentrations of the drug, or metabolites from the drug, to decrease below concentrations that FDA considers safe for human consumption.

The WDT are typically established with small sample sizes (generally less than 30 animals), which can a limit the determination of adequate times when the percentage of acceptable animals with residue is small, such as the 1% limit utilized by FDA. The FDA uses a regression approach to estimate a 99^{th} percentile of a population with a 95% confidence level. (Concordet et al., 1997). With differences in drug depletion curves, this may not always be the best method for determining WDT. The European Union (EU) doesn't think that statistical methods are a sufficient way to evaluate WDT. They establish WDT when all tissues from observed animals fall below the acceptable minimum residue level (MRL).

The most common causes of illegal residues are people ignoring the required WDT, poor record keeping that does not allow for identification and tracking of WDT, and administration of the wrong drug or the wrong dosage. Administering a drug in an extralabel route can result in delayed or incomplete absorption and lead to an increased WDT. In addition, the formulation of a drug can affect how long it can be detected in certain tissues, consequently changing the WDT (KuKanich et al., 2005).

In the U.S., tilmicosin is labeled for a 28 day WDT after receiving a subcutaneous dose of 10mg/kg. In a study by KuKanich et al., (2005) the drug was administered to cattle, and biopsy samples were taken from the site of injection, muscle, liver, kidney and fat at different times to determine the residue depletion time using HPLC. A WDT of 34 days was established such that all tissues except the subcutaneous injection site were below the MRL. This study also showed that administering tilmicosin by extralabel route intramuscularly left residues in the muscle samples at injection site for over 56 days. The

Europeon Medicines Agency (EMA) recommends that if the target tissue of a drug is muscle, then the regulatory authorities should set the WDT on the MRL for muscle (Jiang et al., 2006). Differences in testing and regulations for different countries complicate the use of antimicrobials, especially in animals that may be slaughtered for other countries.

Veterinary drugs that are administered intramuscularly or subcutaneously may require additional consideration for residue issues at the site of injection (Reeves et al., 2007). The quality issue related to injection sites has been addressed by programs like Beef Quality Assurance (BQA; http://www.bqa.org), which has helped to reduce injection site lesions in beef rumps from 21% in 1991 to 2.1% in 2000 (National Beef Quality Audit, 2000). A significant effort to approve subcutaneous labels for many new products for cattle has had a large impact on product quality and likely residue. Although the industry is making great improvements in quality there is not reliable data for exposure to injection site residues at this time.

Residue tests

A good residue-screening test needs to be fast, inexpensive, and sensitive enough to detect residues in accordance with the established MRL. Three bacterial inhibition tests have been developed by FSIS (http://www.fsis.usda.gov) to screen kidneys from food animals at slaughter for residues (Korsrud et al., 1998). There is no screening test currently available to detect the MRL's of all approved drug classes of antibiotics. Every test has limitations for specificity, sensitivity, or ease of use at a slaughter facility.

The Swab Test On Premises (STOP) uses tissue that is macerated with the swab, the swab is then incubated on medium inoculated with *Bacillus subtilis* and with a disk containing an antibiotic for 16-24 hours (Korsrud et al., 1998). The Calf Antibiotic and Sulfa Test (CAST) uses kidney fluid collected by making an incision in the kidney and absorbing fluid, placing the collection with *Bacillus megaterium* in medium and incubating for 16-24 hours. For both the STOP and the CAST, a zone of inhibition around the swab demonstrates the presence of a microbial inhibitor in the collected sample. The Fast Antimicrobial Screen Test (FAST) uses *B. megaterium* and the same medium as the CAST test. However, the medium is supplemented with dextrose and bromcresol purple, which allows the bacteria to grow at a faster rate, thus decreasing the required incubation time from 16 to 6 hours. None of these approved screening tests in the U.S. are sensitive enough to detect chloramphenicol or sulfa drugs. The Live Animal Swab Test (LAST) screens urine and plasma antimortem for drug residues. The LAST was adapted from STOP assay but using a higher concentration of *Bacillus subtilis* spores in the assay.

The Premi Test (Stead et al., 2004) was developed based on bacterial inhibition with the growth of *Geobacillus stearothermophilus,* which is sensitive to several different antibiotics (Schneider et al., 2008). The development and validation of the test proves that it is capable of detecting multiple sulphonamide residues represented in tissues at one-half to one times the MRL. The Premi Test can also distinguish the β-lactams successfully. Because the test uses *G.stearothermophillus* and shows a higher sensitivity towards Gram-positive antimicrobial compounds, it does not detect the aminoglycoside (gentamincin, lincomycin, neomycin, streptomycin), or the phenicol (chloramphenicol,

thiamphenicol) drug classes. The Premi test is sensitive to the macrolide class. Because of the inability to detect some drug classes it is important to be able to distinguish the possible drug residues present before testing.

Penicillin is the drug most often misused, and a preslaughter test using an easily collected body fluid from the treated animal to predict if the residue level is low enough for slaughter would be beneficial to the food animal industry. Chiesa et al. (2006) looked at animals treated with penicillin and evaluated correlations between kidney tissue samples collected by laparoscope, plasma, and urine. They showed a fivefold lower drug concentration in plasma compared to kidney tissue. A plasma penicillin concentration of <0.4 ng/ml can be used as an indicator with 95% confidence that 99% of all the kidneys will be below the accepted MRL at slaughter. The average penicillin in urine was approximately 10 times greater than the kidney cortex concentration. A urinary penicillin concentration of <140 ng/ml can be used with 95% confidence that 99% of all the population will be below MRL at slaughter.

Three residue-screening tests, the Four Plate Test (FPT), Screening Test for Antibiotic Residues (STAR), and the Premi Test, were compared (Janosova et al., 2008) to evaluate their ability to detect 10 different sulphonamides. The MRL is only effective if a good screening program is in place. This study showed that the Premi Test was the most sensitive to the sulphonamides.

The most widely used class of antibiotics is the β-lactams, which include penicillins and cephalosporins. Several different residue tests have been developed for

analyzing samples that may contain these antibiotics. A simple, rapid, sensitive, and specific test has been developed to confirm 10 of the β-lactam antibiotics from bovine kidney samples (Fagerquist et al., 2005). Using a solvent extraction, dispersive solid-phase extraction and liquid chromatography-tandem mass spectrometry (LC/MS/MS) for confirmation and quantification. Bovine kidney samples received from FSIS were tested for β-lactams. In the 23 samples that were tested with this method, 70% of the β-lactams tested positive in samples except for desfuroylceftiofur cysteine disulfide (DCCD), which is a metabolite of ceftiofur. The recovery rate for DCCD was 58%.

The ideal screening test should give a reliable result close to the MRL level with few, if any, number of false positive or negative samples (Schneider et al., 2008). A large number of false positives leads to excessive tests to confirm the results, and if no follow-up testing may lead to monetary loss to the plant or the owner. In contrast, false negative tests have the potential for unsafe animal products entering the food supply.

A study, done by Schneider et al., (2009) used 235 carcasses that were retained during slaughter and sampled by FSIS inspectors. Beef kidney juice, and serum was collected to evaluate the FAST, Premi, and KIS® screening tests as well as Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for residue confirmation. Each of the samples was split and tested simultaneously with FAST, Premi, and KIS® screening tests. A separate analysis was also done on each sample for aminoglycosides and other antibiotics using LC-MS/MS. The screening tests were easy to perform, and the FAST test was easy to read with zones of inhibition (Korsrud et al., 1998). However, the color changes in Premi and KIS® tests were sometimes difficult to interpret. Most of the

carcasses sampled (196 out of 235) were negative for any antibiotic residues. All three of the screening tests detected 2 samples with dihydrostreptomycin, and the Premi and KIS® tests detected a sample with sulfamethazine while the FAST did not. LC-MS/MS detected 3 antibiotics with no U.S. FDA approved tolerance listed for beef cattle. The Premi test found a sample with gentamicin and kanamycin in serum, however none of the screening tests detected these antibiotics in kidney juice.

This study demonstrates that no specific test currently available is reliable at detecting all antibiotics at MRL (Schneider et al., 2009). The KIS® and Premi both had a number of false positives in both the kidney juice and serum samples, which increases laboratory testing requirements and expense. In the case of mixtures of antibiotics, LC-MS/MS showed an advantage in identification and quantitation. The serum samples were variable in this study, which related to the hydration status of the animal with shipping. Serum may be less variable for antemortem screening on the farm in most consistently hydrated animals.

Approximately one third of the 9.15 million dairy cows in the U.S. are slaughtered annually, which makes up about 18% of U.S. ground beef (USDA-FSIS Audit Report 24601-08-KC). Cull dairy cows and veal calves are the most high-risk animal groups to contain violative residues. When an animal tests positive with a KIS® test in a slaughter plant, samples of liver, muscle, and kidney are shipped frozen to the FSIS Technical Support Laboratory to determine the type and amount of drug residue present.

Ceftiofur (Payne et al., 2004) which is used to treat cattle for respiratory disease, foot rot, and mastitis is rapidly converted to desfuroylceftiofur and desfuroylceftiofur related metabolites. The parent compound is not detected in milk or tissue. Payne et al., 2004 evaluated the regulatory concern that the ceftiofur metabolites were being incorrectly identified during rapid screening tests. HPLC was used to detect ceftiofur metabolites in dairy cattle. A high and low dose group of dairy cows were treated and slaughtered following recommended withdrawal times. Liver, kidney, and diaphragmatic muscle was collected and analyzed for ceftiofur and metabolites. The kidney residues were considerably higher than liver or muscle. Most false positive tests were from kidney or liver samples, which had been frozen for shipment and then thawed for analysis. Screening assays were run on frozen samples, so the effect of freezing cannot be compared at this time. Future research to evaluate the cause of false positives in frozen samples could be valuable. The false positive effect was not present in diaphragm tissue.

Four laboratories compared samples of swine and bovine kidney and muscle and bovine milk (Hornish et al., 2003). The sample results were relatively consistent across samples. The methods were reliable for detection and quantitation of ceftiofur and its related metabolites in all samples.

Microbial inhibition tests are usually used for detecting antibiotic residues because they are easy to run and inexpensive. In looking at the development of different tests over the years, it is hard to comparatively evaluate them based on published literature alone because different tissues, tests, and procedures are used. Chemical tests have been considered too specific and expensive for screening but HPLC is capable of

detecting multiple antibiotics and is cost effective when considering the savings of forwarding samples and waiting for results (Pikkemaat et al., 2009). There is still a need for a quality-screening test to detect residues in slaughter facilities.

Gentamicin is an effective and inexpensive antibiotic for treating gram-negative bacterial infections in humans. Most of the antibiotic is rapidly metabolized and eliminated from the animal in the first 48 hours, but for gentamicin a small residue remains in the kidney cortex for months making it unacceptable for use in fed cattle or dairy animals. The Chiesa et al., (2006), study looked at blood and urine samples taken from steers that received three doses of 4 mg/kg gentamicin given intramuscularly on the same day. Kidney tissue was also collected by laproscopic surgery as well as at slaughter. The plasma levels of gentamicin were not detectable by day 3 post-treatment, but the urine samples remained positive for 75 days before the concentration declined to an undetectable level. The correlation between urine and kidney tissue suggested a relationship of 1:100. Therefore, a test that is sensitive enough to detect urine gentamicin concentration of 1 ng/ml is equivalent to a 100 ng/g concentration in for kidney tissue.

Flunixin Meglumine

Flunixin meglamine was originally labeled for horses in the U.S. and had limited residue data for use in food animals. However, flunixin is now approved for food animals. The FARAD recommends a WDT of 72 hours for milk, and 10 days for meat after intramuscular injection with the data collected for Flunixin. (Damian et al., 1997)

The use of NSAIDs can improve an animal's outcome to a disease. A survey (Kopcha et al., 1992) of 2000 food animal veterinarians in the United States found that 93% used NSAID, with 57% of them having used a NSAID more than once a week. Flunixin meglamine is the most commonly used, although none of them are approved for food animal use. Approximately 88% of the veterinarians said when they used an NSAID with an antibiotic, the recommended withdrawal time for meat and milk was based on the antibiotic withdrawal time. The potential for residue is small when flunixin is used with an antibiotic.

Flunixin has been approved for use in non-lactating cattle by intravenous injection. The FDA has a withdrawal time of 4 days for meat (KuKanich et al., 2005). FARAD recommends an extralabel milk withdrawal of 72 hours and a meat withdrawal of 4 days based on published milk residue depletion studies. Flunixin causes excessive injection site lesions so it is not recommended for the product to be given intramuscularly or subcutaneously due to inflammatory response and increased WDT (Haskell et al., 2003). Administering flunixin intramuscularly causes tissue damage, inflammation, and slow or incomplete absorption, which can result in illegal residues.

The FDA has approved 1.1 to 2.2 mg/kg of flunixin given intravenously and WDT of 4 days for meat and WDT of 36 hours for milk (Smith et al., 2008). The American Medicinal Drug Use Clarification Act (AMDUCA) does not consider convenience of administration an acceptable reason for extralabel use of a drug. Cattle producers are not necessarily skilled at giving medications intravenously. Administering flunixin intramuscularly is very irritating to tissue and increases creatinine kinase from

baseline levels of 86 U/L to 136 U/L. Because of the tissue irritation, FARAD has recommended a WDT of 30 days for meat when flunixin is administered intramuscularly. There is limited data for subcutaneous administration of flunixin so a WDT has not been established. FARAD recommends an 8-day WDT for meat with oral administration and 48 hours WDT for milk. However, flunixin granules or paste are not protected under AMDUCA rules so only approved formulations should be used in cattle.

Flunixin is considered a drug with high regulatory concern because of the common extralabel routes of administration people use in cattle. Producers should follow label directions if possible. If an extralabel route is used an extended WDT should be recommended for meat and milk.

Ceftiofur

Ceftiofur is an antibiotic with broad-spectrum use that can be used to treat respiratory disease in cattle. It has a low level of toxicity and does not have a withdrawal time following treatment. After injection, it is converted into free and protein bound metabolites. These metabolites have antimicrobial activity and can be detected by residue tests. It is important to be able to distinguish between this β-lactam, which has no residue. At this time, there is no maximum residue limit for ceftiofur metabolites in tissues in the U.S. (Moats et al., 1998).

There are various screening tests to detect residues in animal tissues. The USDA-FSIS uses a 7-plate assay test, but some antibiotics are more difficult to detect.

Penicillinase is used in all but one of the plates to detect the β-lactam antibiotics from other drug classes. The β-lactam antibiotics, which include the cephalosporins, are resistant to degradation from penicllinase so they are not identified by this residue-screening test. They can be degraded by other β-lactamases. A chromatographic procedure has been developed to identify different penicillins in tissues, but it cannot identify the cephalosporins or their metabolites in tissues.

The two cepahalosporins currently approved for use in food animals in the U.S. are ceftiofur and cephapirin (Moats et al., 1998). The metabolites of these antibiotics can be identified by rapid screening assays.

Chapter 3

Development and Model Testing of Ante-Mortem Screening Methodology to Predict Prescribed Drug Withholds in Heifers

ABSTRACT

A simple, cow-side test for the presence of drug residues in live animal fluids would provide useful information for tissue drug residue avoidance programs. This work describes adaptation and evaluation of rapid screening tests to detect drug residues in serum and urine. Medicated heifers had urine, serum, and tissue biopsy samples taken while on drug treatment. Samples were tested by rapid methods and high-performance liquid chromatography (HPLC). The adapted microbial inhibition method, kidney

inhibition swab (KIS®) test, was useful in detecting sulfadimethoxine in serum and its response correlated with the prescribed withdrawal time for the drug, 5 to 6 days post treatment. The lateral flow (LF) screening method for flunixin and beta-lactams, adapted for urine, was useful in predicting flunixin in liver detected by HPLC, 96 hours post treatment. The same adapted methods were not useful to detect ceftiofur in serum or urine due to a lack of sensitivity at the levels of interest. These ante-mortem screening test studies demonstrated that the method selected, and the sampling matrix chosen (urine or serum), will depend on the drug used and should be based on animal treatment history if available. The live animal tests demonstrated the potential for verification that an individual animal is free of drug residues before sale for human consumption.

Many antibiotics and medications used in animal treatment have a withdrawal time established by the U.S. FDA to ensure that drug residues, a concern for the meat industry, do not exceed the set tolerance levels. The most recently published 2009 USDA-FSIS Residue Program Data Report listed 135,389 Inspector-In-Charge-Generated (IICG) residue samples from 43,142,500 beef and dairy cattle inspected that year at harvest (19, 20). Of these samples, 1306 contained violative antimicrobial residues and 327 violative flunixin residues (19). Two classes of antimicrobials comprised over half of the documented violative antimicrobial residues (beta-lactams, including ceftiofur and sulfa drugs, such as sulfadimethoxine). While the violative residue rate seems small, violative residues are unacceptable. Management of carcasses that contain violative residues is costly to the USDA, the meat packing industry, and the producers involved. Most important may be the loss of consumer confidence in foods of animal origin. A simple pre-harvest, cow-side test to verify the presence of violative drug

residues in live animal fluids would provide useful information for avoiding drug residues in tissues before the animal is sold for human consumption.

In October 2009, the USDA-FSIS selected Charm Science Inc. Kidney Inhibition Swab (KIS®) test as the antibiotic residue screening test for ruminants in U.S. packing plants (17, 18). KIS® test sensitivity in spiked swine tissue extracts were reported in 2004 (13). In 2009, the method was re-evaluated with beef and swine using drug spiked swabs; the results are shown in Table 1 and compared to tissue tolerances.

Testing live animal fluids by adapting antibiotic screening methods such as the KIS® test, used by the USDA-FSIS for antibiotic residue screening in beef and swine processing plants (1,4), or the related Delvotest™, used by the U.S. Food and Drug Association (FDA) to screen for beta-lactam drug residues in milk, would make it possible for producers and veterinarians to identify or predict livestock that might be considered a high risk for containing a violative antibiotic residue in tissue (6, 8, 9). In addition, there is no preharvest inhibition-based screening test currently available to detect violative residue levels of flunixin and beta lactams such as ceftiofur in cattle prior to market (14). Some inhibitory methods for detecting drug residues have been demonstrated effective in serum (15, 16); however data that correlates serum and urine detection to tissue tolerance levels is limited. Randecker *et al.* evaluated serum and urine as predictor of sulfadimethoxine in swine tissues in 1987 (9). Chiesa *et al.* evaluated correlations between kidney tissue concentrations and urine or plasma that would lead to the development of rapid screening tests for penicillin (3). Evaluation of *Geobacillus stearothermophilus*-based screening tests, such as the KIS® test, for their ability to predict

violative levels of drug residues in ante-mortem animal urine and serum samples prior to harvest could provide a tremendous benefit to the industry.

MATERIALS AND METHODS

Animals and procedures. All animal procedures were reviewed and approved by the U.S. Meat Animal Research Center (USMARC) Animal Care and Use Committee.

Blood, urine and tissue samples. All animals were housed on center at the USMARC Feedlot and procedures were performed under veterinary guidance. A preliminary study utilized twelve feedlot heifers to test all the collection and laboratory procedures. A larger study later involved twelve heifers in each of the three treatment groups and the control group. The heifers weighed between 340-430 kg, were fed a corn-based finishing ration and had not received any antibiotic treatment for the previous 60 days. Heifers were randomly assigned to the experimental groups. Urine and blood were collected from each animal before administration of their assigned medication (Day 0). The heifers were weighed and treatment groups received ceftiofur sodium (2.2 mg/kg BW, IM), sulfadimethoxine (55 mg/kg BW, IV), or flunixin meglamine (2.2 ml/kg BW, IV). Twenty-four hours after administration, urine and blood was collected from each animal. In addition, 24 h post-treatment a renal biopsy was collected from control heifers and heifers receiving ceftiofur by a peri-cervical approach with a 4 mm biopsy punch. At 24 h post-treatment for the heifers that received flunixin meglumin and the control group, a liver biopsy was performed between the 10^{th} and 11^{th} ribs using a 4 mm biopsy punch. On day 4 of the study, a renal biopsy was collected as described above from heifers

treated with sulfadimethoxine and the control group. Urine and serum was collected every 24 hours up to 24 hours past the FDA-approved medication withdrawal time for each treatment drug At the prescribed withdrawal time for each treatment, urine and blood were again collected from each heifer. Each of the serum and urine samples were split and tested at both USMARC and at Charm Sciences Inc. for comparison and confirmation of results.

KIS® test modifications. All urine and serum samples were examined by each of the following two minor modifications of the KIS® test. No significant differences in results were obtained between the two modifications.

Modification I: (USMARC, Clay Center, NE). Serum samples were centrifuged (1600 x g, 10 min). A sample of urine or serum (500 µl) was combined with a KIS® urine tablet in a microtube. The microtube was capped and vortexed (10 s), inverted to ensure tablet was dissolved, and vortexed again (10 s). A KIS® swab absorbed the mixture and was incubated for 4 hours at 30º C. All urine samples were tested similarly without sample centrifugation.

Modification II: (Charm Sciences, Inc., Lawrence, MA). Serum samples were pre-centrifuged (1200 x g, 3 min) and supernatant (100 µl) was added to a buffering/nutrient tablet (Charm Sciences, Inc.). The mixture was dissolved for 30 minutes, absorbed onto a KIS® swab and incubated (4 h) at 30º C. Urine samples were tested similarly with no sample pre-centrifugation.

Flunixin and beta-lactam (FLUBL) lateral flow (LF) modification. Urine and pre-centrifuged serum samples were tested using a modified lateral flow test, LF-FLUBL (Charm Sciences Inc.) approved for screening raw milk in the U.S. (5, 14). Samples (100 µl) were used to rehydrate a LF-urine/serum tablet (Charm Sciences Inc.). A sample extraction swab (Charm Sciences Inc.) adapted for kidney testing, consisting of a sampling swab and 750 µl buffer, was used to absorb the tablet mixture into the swab and dilute into the buffer. Extract buffer (300 µl) was added to the LF-FLUBL test strip and the test was performed and results read in a ROSA reader (Charm Sciences Inc.) according to instructions for testing milk and kidney (5).

High Performance Liquid Chromatography (HPLC). HPLC system consisted of a Waters Alliance HT (Waters 2795; Waters Corporation, Milford, MA) liquid chromatography system equipped with a diode array multiple wavelength detector (Waters 996) and a Shimadzu column oven (CTO-10AS VP; Shimadzu America, Columbia, MD) set at 30°C for all sample analysis. Sulfadimethoxine analysis was performed using a Phenomenex Gemini 5µ C18 110A, 250 x 4.6 mm column. HPLC for sulfadimethoxine followed the method of Primus *et al.* (11). Urine and serum, 1 ml volume, were substituted in the method to yield limit of detection (LOD) of 250 ppb for serum and 500 ppb for urine. Ceftiofur analysis was performed using a Thermo Scientific Hypersil BDS C-18, 250 x 4.6 mm column (Waltham, MA). HPLC for ceftiofur was performed following a Desfuroyl Ceftiofur Acetate (DCA) derivative quantitation method (6). The method was adapted by substituting 1ml serum or urine for the milk portion in the procedure to yield LOD of 5 ppb. Flunixin analysis was performed using a Phenomenex Hypersil 5µ ODS (C18; Phenomenex, Torrance, CA), 120A, 250 x 4.6 mm

column. Flunixin in serum and urine was analyzed by method developed by Königsson et al. and had an LOD 20 ppb (7). Flunixin glucuronide metabolite in urine only was detected by hydrolysis to free flunixin by strong base and addition of β-glucuronidase (2) and the product was analyzed flunixin as described (7), except samples were frozen at -80°C instead of -20°C.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). All tissue biopsies were forwarded to the USDA- Agriculture Research Service (ARS) Eastern Regional Research Center for LC-MS/MS analysis. Biopsy samples were frozen and transferred to a small mortar and pestle and manually ground in the presence of 80/20 acetonitrile/water (4 mL), along with sufficient internal standard [sulfadoxine (USP) for sulfadimethoxine analysis, and flunixin-d_3 (Toronto Research Chemicals, Toronto) for flunixin analysis] to provide the desired level (500 ppb for sulfadoxine, 100 ppb for flunixin-d_3). The resultant mixture was tranferred to a disposable 15 mL tube and centrifuged (3716 x g, 5 min), after which the supernatant was decanted into a disposable centrifuge tube containing C-18 sorbent. After vortex mixing (15 s) and centrifugation, a portion (3.0 mL) of the supernatant was transferred to a glass tube and evaporated to <0.5 mL volume using a Turbovap LV (Zymark, Hopkinton, MA) under stream of N_2 at 40°C. After addition of water to 0.5 mL, samples were mixed and transferred to a PVDF filter vial (0.2 µm, Thomson, Oceanside, CA) for LC-MS/MS analysis.

LC-MS/MS instrumentation and chromatography column used have been previously described, along with general MS parameters, except for use of 100 ms dwell time (21). Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in

acetonitrile (B). Gradient elution was employed (80% A - 0% A, 10 min). After an additional 1 min at 0% A, the column was re-equilibrated to intial conditions (6 min). Flow rate was 400 µL/min. Specific analyte MS parameters were as follows: sulfadoxine transitions monitored: m/z 310.9-156.0 and 310.9-92.0, declustering potential (DP): 46 V, focusing potential (FP): 170 V, collision energy (CE): 25 V and 43 V, collision cell exit potential (CXP): 12 V and 8 V; sulfadimethoxine transitions monitored: m/z 311.0-156.0 and 311.0-108.1, DP: 36 V, FP: 160 V, CE: 29 V and 43 V, CXP: 12 V and 8 V; flunixin transitions monitored: m/z 297.0-278.9 and 297.0-263.9, DP: 36 V, FP: 110 V, CE: 33 V and 47 V, CXP: 16 V and 16 V; flunixin-d3 transitions monitored: m/z 300.1-282.1 and 300.1-264.0, DP: 36 V, FP: 140 V, CE: 31 V and 45V, CXP: 26 V and 24 V. Quantification of analytes utilized matrix matched calibration curves.

RESULTS AND DISCUSSION

Urine and serum incurred samples were analyzed by HPLC in order to establish a concentration for the analytes in these matrices. Ceftiofur incurred serum and urine samples were then also analyzed by the KIS® and LF assays, flunixin incurred samples were analyzed by the LF assay, and sulfadimethoxine incurred samples were analyzed by the KIS® assay. A small scale preliminary dosing study was conducted to test the experimental procedures. In the preliminary study, ceftiofur-dosed heifers provided urine and serum samples that were negative at all time periods in the KIS® and LF assays. Development of LF assay continued to try to improve the limit of detection for the second study. Preliminary study results for flunixin and sulfadimethoxine were comparable to the main study and reported here.

Ceftiofur incurred serum and urine analysis results for the main study are shown in Table 2. Levels of ceftiofur in these samples as measured by HPLC ranged from 154-3680 ppb in urine and 207-1280 ppb in serum (24 h) to <_5 ppb (LOD) at the intramuscular or subcutaneous injection WDT (96 h). The KIS® and LF assay results for these samples are also indicated in Table 2. One can see that the KIS® results ranged from 7/12 positive in urine and 2/12 positive in serum (24 h) to 0 KIS® positive samples at 96 h. The LF results ranged from 10/12 positive in urine and 6/12 positive in serum (24 h) to 0 LF positive samples at 96 h.

The observed trend in assay response is generally what is desired, and all samples were negative at the WDT; however, a fundamental problem is that the tolerance is 400 ppb in kidney, and the KIS® and LF LOD is 600 ppb in kidney and serum. Thus, neither test will likely be sensitive enough to detect violative samples corresponding to the range of 400-600 ppb in tissue. Furthermore, the levels in urine and serum may very well be lower than in kidney, and thus, more difficult to detect.

Flunixin incurred serum and urine analysis results for the main study are shown in Table 3. Levels of flunixin in these samples as measured by HPLC ranged from 290-1940 ppb in urine, with lower levels in serum (30-830 ppb, and 6/12 samples <20 ppb) at 24 h. At the 96 h withdrawal time, flunixin levels in urine and serum as measured by HPLC were < LOD with the exception of 3 urine samples (30-70 ppb).

As a NSAID, flunixin will not be detected by an antibiotic inhibition assay such as KIS®. Thus, an important part of this study was to develop a live animal screen for

flunixin. The LF assay was investigated, and results for the flunixin incurred urine and samples are indicated in Table 3. At 24 h, all urine samples were LF positive. The maximum dilution which still produced a positive LF assay result was 1:25 for 2 urine samples, and 1:50 for the remaining 10 urine samples. As the LOD for the LF assay is 30 ppb in both urine and serum, the urine dilution range for a negative result corresponds to a range estimate of >750 ppb and >1500 ppb, respectively for these urine samples. At 24 hr, all serum samples were also LF assay positive for flunixin. The maximum dilution which gave a positive LF assay result was 1:25 for 4 serum samples, while the remaining 8 samples required no dilution. This corresponds to an estimate of >750 ppb and >30 ppb flunixin for these serum samples, respectively. At the 96 h required WDT, all urine and serum samples were negative for flunixin using the LF assay, as would be desired for samples expected to be below tolerance levels at that time. In data not reflected in Table 3, the modified LF method with multiple replicates of the untreated animal samples had a false positive result that was more frequent with serum samples (8%) than in urine samples (>4%).

The tissue biopsy results taken from livers at 24 hours are shown in Table 4 compared to the LF estimates of ppb and HPLC quantified urine samples. Urine, as opposed to serum, was selected as the comparator in this table because of the higher detected levels of drug and the longer clearance times that were consistent with the prescribed tissue withholding time. There were 4 liver samples that exceeded tissue tolerances of 125 ppb and the remaining samples had detectable levels ranging from 24-76 ppb. The recommended animal withhold time of IM flunixin is 4 days and these 24 h results would indicate that the liver levels should be well below tolerance after 3

additional days withheld. The drop in detectable urine levels at 48 h compared to 24 h support that there is a rapid clearing of the drug from the animals.

As the second most detected drug in IICG samples (18), a flunixin assay must be used concurrently with the KIS® assay for antibiotics to be detected with such a high frequency in national sampling programs. Urine, as opposed to serum, testing using modified-LF at the 1:50 dilution was useful in predicting flunixin levels above and below liver tolerance of 125 ppb. The method was cross-reactive to the major urine metabolite, flunixin-glucuronide, and therefore detected 75% of samples as positive at 24 h with the KIS® test, when 33% of the samples exceeded tolerance in the liver biopsy. At 48 h, 2 days earlier than the recommended tissue withhold, the 1:50 dilution passed all samples as negative, but the undiluted urine samples were still 75% positive indicating urine may be useful to detect proper drug withhold time. At 48 h withhold, there was a significant reduction in the detected urine levels of flunixin-glucuronide that should correlate to a significant reduction in flunixin in tissue. More research is needed to make this determination. The 72-h and 96-h urine samples were 50% positive by LF undiluted assay, when the recommended withhold time of this drug treatment is 96 h. This indicates that the screening method may be useful to identify treated animals up to their withhold time even though the LF by the higher dilution of 1:25 or 1:50 is more predictive of volatile tissue levels,

Data from the main study for sulfadimethoxine is shown in Table 5. Levels of sulfadimethoxine in urine samples as measured by HPLC ranged from 40 to 11,100 ppb in urine at 24 h, with 8/12 of these samples responding negatively in the KIS® assay.

These levels dropped off rapidly in urine, and at 48 h, all samples tested negative by KIS®. Sulfadimethoxine residues in serum were present at much higher levels than in urine, ranging from 44,300 to 139,000 ppb by HPLC at 24 h, and with all samples at this time being positive by KIS®. LC-MS/MS of biopsy samples taken 4 days post-dosing show that kidney levels generally exceeded 100 ppb tolerance. The higher kidney SDM levels on day 4 correlated with KIS® serum test positive results on days 4, 5 and 6. KIS® test serum negative results occurred at 96 h with kidney LC-MS/MS levels of 150 ppb, 99 ppb, and 302 ppb.

The KIS® test was very useful to correlate the detection of sulfadimethoxine in tissue when serum, but not urine, was used as the live animal predictive sample. Serum samples were still positive on day 4 when tissue biopsy samples were taken and support that the tissue were exceeding tolerance. Urine samples, however, tested negative after the initial 24 h and would not be useful in predicting sulfadimethoxine in tissue. It is interesting to note that kidney biopsies taken on day 4 (just 24 h prior to the recommended WDT of 5 days) from the heifers receiving SDM were still well above the 100 ppb approved drug tolerance level set forward by the FDA see Table 5. Several of the animals tested on day 5 and 6, post-withhold time, were still serum positive for sulfadimethoxine. This suggests that the KIS® serum method, which had 25% of serum samples on day 6 test positive, could either be erring on the side of safety in predicting tissue above tolerance or that the tissues were in fact still exceeding tolerance. More research is needed to make this determination.

In conclusion, results from this study show that ante-mortem testing of animal fluids as a predictor of tissue residue is dependent on the drug with which the animal is treated. Residues of sulfadimethoxine in tissue were able to be predicted with serum, but not urine, when tested using an inhibition test like the KIS® test. Residues of the anti-inflammatory drug flunixin were able to be predicted with a LF method of urine samples, but not serum samples. Neither serum nor urine was useful in detecting ceftiofur residues due to the method's insensitivity to levels achieved in serum and urine. Further drug studies are needed to understand the drug residue relationships between tissue, urine, and serum to develop future pre-harvest screening tests.

ACKNOWLEDGEMENTS

We would like to thank Dr. Gary Rupp for his technical assistance with the kidney biopsies and Janel Nierman for her secretarial assistance. We also would like to thank the USMARC feedlot crew, in particular Chad Engle, B.J. Johnson, Randy Scott, and Gail Roubinek for their dedication to animal care and their assistance in the handling and management of the heifers in this study.

REFERENCES

1. Anadon, A. and M. R. Martinez-Larranaga. 1999. Residues of antimicrobial drugs and feed additives in animal products: regulatory aspects. *Livest. Prod. Sci*. 59:183-198.
2. Brady T., A. Kind, W. Hyde, M. Favrow, and D. Hill. 1998. Isolation Purification, and structural characterization of flunixin glucuronide in the urine of greyhound dogs. *Drug Metabolism and Disposition* 26:294-298.
3. Chiesa O. A., J. von Bredow, M. Smith, D. Heller, R. Condon, and M. H. Thomas. 2006. Bovine kidney tissue/biological fluid correlation for penicillin. *J. Vet. Pharmacol. Therap*. 29:299-306.
4. Dey, B. P., M. A. Thaler, and F. Gzozdz. 2003. Analysis of Microbiological Screen Test Data for Antimicrobial Residues in Food Animals. *J of Environmental Science and Health*, 38-3, 391-404.
5. Douglas, D., K. Banaszewski, R. Juskelis, F. Al-Taher, Y. Chen, J. Cappozzo, and R. S. Salter. 2012. Validation of a Rapid Lateral Flow Test for the Simultaneous Determination of β-Lactam Drugs and Flunixin in Raw Milk. *J. Food Protection* 75:1270-1277.
6. Hamlow, P. 1994. Determinative Method for Ceftiofur-Related Residues in Bovine Milk (HPLC-DCA). *Pharmacia & Upjohn Animal Health*, 1-43. Method is on file with the Center for Veterinary Medicine, Food and Drug Administration, 7500 Standish Place, Rockville, MD 20855.

7. Königsson K., K. Törneke, I. V. Engeland, K. Odensvik, and H. Kindahl. 2003. Pharmacokinetics and pharmacodynamic effects of flunixin after intravenous, intramuscular and oral administration to dairy goats. *Acta Vet. Scand*. 44:153-159.
8. Korsrud, G. O. and J. O. Boison. 1998. Bacterial inhibition tests used to screen for antimicrobial veterinary drug residues in slaughtered animals. *J. AOAC International* 81:21-24.
9. Korsrud, G. O. and J. D. MacNeil. 1988. Evaluation of the swab test on premises, the calf antibiotic and sulfa test, and a microbial inhibitor test with standard solutions of 22 antiobiotics. *J. Food Prot.* 51:43-46.
10. NADA 141-288. 2008. Freedom of information summary New Animal Drug Application NADA 141-288 EXCENEL RTU EZ Sterile Suspension Ceftiofur hydrochloride Sterile suspension for injection Swine and cattle (beef, non-lactating dairy, and lactating dairy). http://ebookbrowse.com/gdoc.php?id=198323907&url=55116a8c6a830fabbb91111c077b9df0. Accessed 18 February 2013.
11. Primus T., S. Jojola, S. Robinson, and J. Johnston. 2007. Determination of Sulfadimethoxine Residues in Skunk Serum by HPLC. *J. Liq. Chromatogr. Rel. Technol.* 30: 2095-2102.
12. Randecker, V. W., J. A. Reagan, R. E. Engel, D. L. Soderberg, and J. E. McNeal. 1987. Serum and Urine as Predictors of Sulfamethazine Levels in Swine Muscle, Liver and Kidney. *J. Food Prot*. 50:115-122.

13. Salter, R. S., M. S. Rossi, and S. J. Saul. 2004. Kidney Inhibition Swab (KIS®) Test. Broad Spectrum Microbial Inhibition Test Meets Tolerances within 3 Hours. Poster presented at the national conference of the 2004 AOAC International, Baltimore, MD.

14. Salter, R. S., D. Douglas, E. Conklin, R. Juskelis, K. Banaszewski, F. Al-Taher,Y. Chen, and J. Cappozzo. 2011. Screening Method for Flunixin and Ceftiofur in Bovine Kidney. Poster presented at the International Association of Food Protection Conference, Milwaukee, MN.

15. Schneider, M. J. and S. J. Lehotay. 2008. A comparison of the FAST, Premi and KIS® test for screening antibiotic residues in beef kidney juice and serum. *Anal. Bioanal. Chem.* 390:1775-1779.

16. Schneider, M. J., K. Mastovska, S. J. Lehotay, A. R. Lightfield, B. Kinsella, and C. E. Shultz. 2009. Comparison of screening methods for antibiotics in beef kidney juice and serum. *Anal. Chim. Acta* 637:290-297.

17. USDA-FSIS Notice 39-09. May 5, 2009. Electronic sample (eSAMPLE); Phase I – Reporting in-plant fast antimicrobial screen test (FAST) results.

18. USDA-FSIS Notice 50-09. July 15, 2009. Using the kidney inhibition swab test (KIS®) test to detect antimicrobial drugs residues in cattle in selected establishments. http://www.fsis.usda.gov/OPPDE/rdad/FSISNotices/43-12.pdf. Accessed 12 November, 2011.

19. USDA-FSIS. 2009a. The 2008 FSIS National Residue Program Data. United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science; published October 2009.

http://www.fsis.usda.gov/Science/2009_Red_Book/index.asp. Accessed 17 March, 2011.

20. USDA-FSIS. 2009b. The 2009 FSIS National Residue Program Scheduled Sampling Plan. United States Department of Agricultre, Food Safety and Inspection Service, Office of Public Health Science; published October 2009. http://www.fsis.usda.gov/Science/2009_Red_Book/index.asp. Accessed 20 March, 2011.
21. Schneider, M. J., K. Mastovska, and M. B. Solomon. 2010. Distribution of Penicillin G Residues in Culled Dairy Cow Muscles: Implications for Residue Monitoring. *J. Agric. Food Chem*. 58:5408-5413.

Table 1- KIS® test sensitivity determined from beef and swine kidney spiked swabs after sampling compared to Tissue Tolerances (T=tissue, K=kidney, L=liver)

Antibiotic Drug	KIS® Detection Level (ppb)	U.S. Tolerance@ (ppb)
Penicillin G	30-40	50 T
Ampicillin	100	10 T
Amoxicillin	100	10 T
Cloxacillin	300	10 T
Ceftiofur*	4000	400 K 250 K (swine)
Cephapirin*	100	100 T
Sulfamethazine	500	100 K
Sulfadimethoxine	250	100 K
Sulfathiazole	250	100 K
Oxytetracycline	3000	12000 K
Chlortetracycline	12000	12000
Tetracycline	1000	12000
Tylosin	400	100 K
Erythromycin	500	100 T
Pirlimicin*	1000	500cow L
Tilmicosin	2500	100 T 7500 L (swine) 1200 L (bovine)
Tulathromycin*	400	3000 K
Neomycin	1000	7200 K
Gentamicin	750	400 K
Streptomycin	10000	2000 K
Dihydrostreptomycin	2000-4000	2000 K
Floramphenicol	10000	2500 L (swine) 3500 L (bovine)
Chloramphenicol	50000	-
Enrofloxacin	25000	100 L (bovine)
Ciprofloxacin	25000	-
Spectinomycin	10000	4000 K
Novobiocin	5000	1000 T
Trimethoprim	1000	-
Virginiamycin	25000	400 K (swine)
Bacitracin^	10000	500 T

* Drugs known to metabolize into multiple forms. Spiked drug sensitivity may not accurately reflect incurred drug level.

^ mIU/ml

@ Tolerance from http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=044c20ff4bcde0eaf632fb124f3bdbcf&rgn=div6&view=text&node=21:6.0.1.1.16.2&idno=21

Table 2- Second incurred ceftiofur study showing HPLC determined part per billion (ppb) ceftiofur at sampling internals[a]

Animal ID#	Urine Samples (ppb)					Serum Samples (ppb)				
	0 h[b]	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
2956	nd	154 (K+, L+)	198	433	nd	nd	375 (L+)	110 (L+)	110	nd
2962	nd	679 (L+)	183 (L+)	28	nd	nd	1008 (L+)	162	12	nd
2983	nd (K+)	1607 (L+)	147	48	nd	nd	207	101	21	nd
2996	nd	1006 (L+)	216	67	nd	nd	744 (L+)	202	15 (K+, L+)	ns[c]
3009	nd	1475 (L+)	151	79	nd	nd	833 (K+, L+)	249	34	nd
3011	nd	339 (K+)	ns (K+)	ns (K+)	ns	nd (L+)	576 (L+)	ns	ns	ns
3033	nd (L+)	718 (K+)	ns	38	nd	nd	819	131	15	nd
3041	nd	676 (K+, L+)	488 (L+)	23	nd	nd	381	345	46	nd
3141	nd	2331 (K+, L+)	49	30	nd	nd	997 (K+, L+)	272	24	9
3198	nd	419 (L+)	551 (L+)	27	nd	nd	631	135	13	ns
3262	nd	3680 (K+, L+)	75	30	nd	nd	620	93	33	nd
3951	nd	332 (K+, L+)	167	47	nd	nd	1280	237	42	nd

[a] K+, KIS positive; L+, lateral flow positive; ND, not detected by HPLC analysis (<5 ppb LOD); NS, no sample for HPLC analysis.

[b]Before treatment with ceftiofur sodium at 2.2 mg/kg of body weight, intramuscularly.

Table 3- Second incurred flunixin study showing HPLC determined ppb at sampling internals[a]

Animal ID#	Urine Samples (ppb)					Serum Samples (ppb)				
	0 h[b]	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
2928	nd	1010 (L+,50)[c]	340 (L+)	160 (L+,10)	nd (L+)	nd	830 (L+,25)	680 (L+,10)	nd	nd
2959	nd	790 (L+,25)	30 (L+)	nd	nd (L+)	nd	nd (L+)	nd	nd	nd
2961	nd	1270 (L+,50)	100 (L+,25)	50 (L+,10)	30	nd	160 (L+,25)	nd	nd	nd
2989	nd	1460 (L+,50)	80 (L+,10)	260 (L+)	70 (L+,10)	nd	30 (L+)	nd	nd	nd
2990	nd	740 (L+,50)	240	nd	nd	nd	nd (L+)	nd	nd	nd
3007	nd	340 (L+,50)	60	nd	nd	nd	nd (L+)	nd	nd	nd
3023	nd	1120 (L+,50)	30	nd	nd	nd	nd (L+)	nd	nd	nd
3024	nd	1550 (L+,50)	120 (L+,10)	60 (L+)	nd (L+)	nd	70 (L+)	nd	nd	nd
3044	nd	290 (L+,25)	ns (L+)	ns	ns	nd	nd (L+)	nd	nd	nd
3153	nd	1770 (L+,50)	220 (L+,10)	30 (L+)	50 (L+)	nd	380 (L+,25)	130 (L+,25)	nd	nd
3227	nd	380 (L+,50)	110 (L+,10)	30	nd	nd	nd (L+,25)	nd	nd	nd
3995	nd	1940 (L+,50)	70 (L+)	60 (L+)	nd (L+)	nd	40 (L+)	nd	nd	nd

[a]L+, laeral flow positive; noted with maximum-fold dilution of sample providing a laeral flow positive response; ND, not detected by HPLC analysis (<20 ppb LOD); NS, no sample for HPLC analysis.

[b]Before treatment with flunixin meglamine at 2.2 ml/kg of body weight, intravenously.

[c]Dilution used to generate a positive response is noted where appropriate: 50 = 1:50, 25 = 1:25, 10 = 1:10, and no number = undiluted sample.

Table 4- Second incurred flunixin study showing urine screening and HPLC flunixin-glucuronide concentrations (ppb) at 24 and 48 hours compared to LC-MS-MS flunixin (ppb) of 24 hour liver biopsy sample

	24 h Post-Treatment			48 h Post-Treatment	
Animal ID#	Liver LC-MS-MS (ppb)[a]	Urine LF Screen (ppb)[b]	Urine Modified-HPLC (ppb)	Urine LF Screen (ppb)[b]	Urine Modified-HPLC (ppb)
2928	74	>1500	1010	30-300	340
2959	24	750-1500	790	30-150	30
2961	45	1500-3000	1270	>750	100
2989	187	>1500	1460	300-750	80
2990	47	>1500	740	<30	240
3007	56	>1500	340	<30	60
3023	60	>1500	1120	<30	30
3024	54	>1500	1550	300-750	120
3044	33	750-1500	290	>30	nd[c]
3153	247	>1500	1770	300-750	220
3227	165	>1500	380	>300	110
3995	150	>1500	1940	>30	70

[a]LC-MS/MS liver samples greater than 125 ppb are violative tissue residue.
[b]LOD of screen = 30 ppb multiplied by largest dilution for positive.
[c]ND, not detected.

Table 5: Sulfadimethoxine determined in urine and serum samples by HPLC from second dosing of heifers compared with LC-MS/MS of kidney biopsy take at 96 h[a]

Animal ID#	Urine Samples (ppb) 0 h[b]	24 h	48 h	72 h	96 h	120 h	144 h	Serum Samples (ppb) 0 h	24 h	48 h	72 h	96 h	120 h	144 h	Kidney (ppb) 96 h
79	nd	9500 (K+)	300	300	nd	nd	nd	nd	100000 (K+)	18200 (K+)	4000	1600 (K+)	1000	300	176
2903	nd	700	100	nd	500	nd	nd	nd	51000 (K+)	15600 (K+)	4000 (K+)	1900	500	200	150
2940	nd	7000 (K+)	700	4000	300	200	nd	nd	75000 (K+)	24000 (K+)	10200 (K+)	2800 (K+)	900	200	ns[c]
2953	nd	760	nd	nd	nd	nd	nd	nd	49400 (K+)	14700 (K+)	5200 (K+)	1600 (K+)	300	200	252
2957	nd	11100 (K+)	600	nd	400	ns	nd	nd	65300 (K+)	19600 (K+)	12200 (K+)	4100 (K+)	1000 (K+)	1300	389
2960	nd	5000 (K+)	nd	nd	nd	nd	nd	nd	85200 (K+)	21000 (K+)	9000 (K+)	4800 (K+)	3200 (K+)	2200 (K+)	384
2968	nd	2100 (K+)	nd	100	nd	100	nd	nd	139000 (K+)	24900 (K+)	9600 (K+)	3000	1400 (K+)	800	302
2980	nd	11100 (K+)	600	1500	40	nd	nd	nd	48200 (K+)	14000 (K+)	5000 (K+)	4700 (K+)	700	650	ns
3034	nd	40	nd	nd	nd	nd	nd	nd	45000 (K+)	15500 (K+)	5000 (K+)	1300 (K+)	700	200	161
3184	nd	1000	nd	nd	nd	nd	nd	nd	44300 (K+)	11500 (K+)	2900 (K+)	700	300	300	99
3913	nd	1000 (K+)	nd	nd	nd	nd	nd	nd	67100 (K+)	22600 (K+)	12000 (K+)	4900 (K+)	3400 (K+)	2200 (K+)	852
3916	nd	5000 (K+)	2200	1300	4200 (K+)	100	nd	nd	83500 (K+)	30700 (K+)	12200 (K+)	5200 (K+)	2600 (K+)	1500 (K+)	804

[a]K+, KIS positive; ND, not detected (urine LOD < 500 ppb and serum LOD < 200 ppb for the HPLC methods used); NS, no sample for HPLC analysis.

[b]Before predose intravenous treatment of 55 mg/kg of body weight.

Chapter 4

Conclusion

National Residue Program

FSIS administers the National Residue Program to ensure that our food supply is safe from veterinary drugs, pesticides, and heavy metals. FSIS works in collaboration with the EPA and FDA. The FDA is responsible for approving drugs for food producing animals and setting the acceptable levels of residues in edible tissue. The EPA is responsible for setting the acceptable levels of pesticides in food producing animals. The FSIS will collect and test suspect animals for residues and follow the guidelines set forth by the FDA and EPA.

The FSIS has two sampling plans. One plan is a scheduled sampling plan, which involves randomly sampling carcasses that have been approved for consumption to determine the prevalence of residues in our food supply. The second plan is an inspector generated plan where the inspector will select carcasses based on several factors which include: 1) signs or symptoms noted in the live animal inspection; 2) pathological conditions or abnormalities found in the carcass; 3) previously documented residue violations by the animal's owner; 4) herd history; and 5) animal identified as a "high risk" type such as dairy or bob veal.

FSIS publishes the "National Residue Program Scheduled Sampling Plans (the Blue Book), and the "National Residue Program Data" (the Red Book) annually to report

the testing results for the national residue program that would have been completed the previous year.

Program Concerns

The March 2010 Audit Report (USDA-FSIS 24601-08-KC) revealed several important limitations in the current National Residue Program. In fact, the first finding states that the FSIS and other responsible agencies need to re-establish the program to accomplish the mission. Coordination between the FSIS, EPA, and FDA has not been working to communicate and resolve issues without the Surveillance Advisory Team (SAT), and the Interagency Residue Control Group (IRCG) acting as forums for their communication.

The audit report also concluded that more residues need to be tested. The EPA requests that the FSIS test for 23 different types of pesticides, of which FSIS tests only a few. FSIS argues that EPA has not provided a list of those with acceptable tolerances such that the test results, even if performed, would be useless. In 2008, FSIS ranked 23 pesticides in the annual sampling plan but tested for only one of the pesticides (USDA-FSIS 2009b). With the limited laboratory resources available for the testing program, FSIS has also stated that if they start testing for additional pesticides they will have to decrease the amount of samples they are currently taking for *Salmonella* and Shiga-toxigenic *E. coli* to accomplish the task.

The FSIS and FDA need to improve testing methods. When a drug is submitted to the FDA for approval, the drug company submits the testing method as part of the new drug application. Once the product is approved, FSIS must follow the testing method

when confirming the presence of residues in meat. Unfortunately, the approved test may not be the best test. FSIS has requested FDA to assist them in evaluating new methods for use in testing. This is called “bridging”, and although the FDA indicated they were willing to help FSIS with this issue, they have worked for over two years to bridge penicillin and it is still not done. FSIS has requested that ceftiofur be bridged but FDA has stated that it would be too difficult to bridge this antibiotic, which is one of the top residues found in cattle to date. If the U.S. would move forward to the European Union system of performance-based testing, new technology could be implemented immediately resolving this testing issue.

Tolerances have not been set by the EPA for many pesticides and heavy metals. Consequently, should the FSIS test for one of them they have no idea what is acceptable and no authority to retain the meat in question. In 2008, the Mexican government rejected a load of meat from the U.S. with levels of copper that exceeds their tolerance levels. Since copper does not have an established tolerance level in the United States, FSIS had no basis to stop the distribution of this meat to Americans. As another example, in 2008 a vigilant cattle producer reported that his cattle had ingested arsenic. The cattle were held until testing could be completed, but if the producer had not identified this situation the meat would have also been distributed.

Results from testing need to be available in a more realistic period. By the time FSIS publishes the Red Book it can be up to 12 months after the results were last received. This delay appears to be a due to bookkeeping procedures where testing results have to be manually analyzed and consolidated, taking scientists and staff an excessive amount of time in preparing and editing results for the Red Book. Moving to a more

efficient electronic means of testing and doing away with the current system of hand writing would provide usable information in a timely fashion for all agencies.

Efficiency in identifying animals with barcodes instead of retained tags is also a concern because the retained tags are issued as a group of four. However, FSIS inspectors often need to identify at least six pieces of carcass so they improvise with other methods of labeling. These other methods are not consistent, and are often difficult to read and follow throughout the testing process. A better barcode labeling system that could rapidly and consistently label all required pieces of carcass that needed to be retained for testing is needed.

Conclusions

There is currently not a simple cow-side test for the presence of drug residues in live animal fluids that would provide verification that an animal is free of drug residues before it is sold for slaughter. Identifying animals with residues before they are sold would help eliminate the possibility of them entering the food chain.

FSIS is changing their sampling plans to test fewer samples for more residues. This response to the audit performed in 2010 is a positive step forward in making meat safer for consumers.

The research project we did at USMARC showed higher levels of sulfadimethoxine in 30% of the heifers that were treated at label recommendations within 24 hours of WDT ending. The rate at which animals metabolize pharmaceuticals varies and the statistical methods that we currently use have different rates of success for

different products. More research needs to be done to address this complex issue facing animal agriculture.

Bibliography

Anadon, A. and M. R. Marinez-Larranaga. 1999. Residues of antimicrobial drugs and feed additives in animal products: regulatory aspects. Lest. Prod. Sci. 59:183-198.

Beran, G. W. 1988. "Use of Drugs in Animals: An Epidemiologic Perspective." *Proceedings of the Symposium on Animal Drug Use – Dollars and Sense.* p. 3-27. Rockville, MD: Center for Veterinary Medicine.

Chiesa, O. A., et al. 2006. Bovine kidney tissue/biological fluid correlation for penicillin. J. Vet. Pharmacol. Therap. 29:299-306.

Chiesa, O. A., et al. 2006. Use of tissue-fluid correlations to estimate gentamicin residues in kidney of Holstein steers. J. Vet. Pharmacol. Therap. 29:99-106.

Concordet, D., et al. 1997. The withdrawal time estimation of veterinary drugs revisited. J. Vet. Pharmacol. Therap. 20:380-386.

Damian, P., et al. 1997. Extralabel use of nonsteroidal anti-inflammatory drugs. J. Am Vet. Med. Assoc. 211:860-861.

Dulin, A. 1996. Determination of minimal detectable levels of antimicrobial drugs on FAST, CAST, and STOP test plates (Document marked "Confidential"). USDA-FSIS Science Section.

Fagerquist, C., et al. 2005. Confirmatory and Quantitative Analysis of β-lactam Antibiotics in Bovine Kidney Tissue by Dispersive Solid-Phase Extraction and Liquid Chromatogrphy-Tandam Mass Spectrometry. Anal. Chem. 77:1473-1482.

Fisch, R. et al. 2000. Withdrawal time estimation of veterinary drugs: extending the range of statistical methods. J. Vet. Pharmacol. Therap. 23:159-162.

Floate, K. D. 2005. Can. J. Vet. Res. 70:1-10.

Gomes, E. R. and P. Demoly. 2005. Epidemiology of hypersensitivity drug reactions. Curr. Opin. Allerg. Clin. Immunology 5:309-316.

Haskell, S., et al. 2003. Update on FARAD food animal drug withholding recommendations. J. Am. Vet. Med. Assoc. 223:1277-1278.

Hornish. R., et al. 2003. Multi-laboratory Trial for Determination of Ceftiofur Residues in Bovine and Swine Kidney and Muscle, and Bovine Milk. J. AOAC Int. 86:30-38.

Janosova, J., et al. 2008. Comparison of the sensitivity of antibiotic residue screening methods to sulphonamide standards and their presumptive identification by para-aminobenzoic acid. Medycyna Wet. 64:663-667.

JECFA (Joint Expert Committee on Food Additives). 1990. Evaluation of certain veterinary drug residues in food. WHO Technical Report Series. 799:37-44.

Jiang, H. et al. 2006. Residue Depletion of Tilmicosin in Cattle after Subcutaneous Administration. J. Agric. Food Chem. 54:5208-5213.

Kopcha, M., J. B. Kaneene, M. E. Shea, et al. 1992. Use of nonsteroidal anti-inflammatory drugs in food animal practice. J. Am. Vet. Med. Assoc. 201:1868-1872.

Korsrud, G. O. and J. O. Boison. 1998. Bacterial inhibition tests used to screen for antimicrobial veterinary drug residues in slaughtered animals. J. AOAC Int. 81(1):21-24.

Korsrud, G. O. and J. D. MacNeil. 1988. Evaluation of the swab test on premises, the calf antibiotic and sulfa test, and a microbial inhibitor test with standard solutions of 22 antibiotics. J. Food Prot. 51(1):43-46.

KuKanich B. R. Gehring, A. I. Webb, A. L. Craignill, and J. E. Riviere. 2005. Effect of formulation and route of administration on tissue residues and withdrawal times. J. Am. Vet. Med. Assoc. 227:1574-1577.

Martinez, U.Z. 2005. Health official: clenbuterol cases rising. Miami Herald, Mexico Edition, 28 November 2005 (http://www.eluniversal.com.mx/miami/15989.html)

Moats, W., et al. 1998. Identification of β-Lactam Antibiotics in Tissue Samples Containing Unknown Microbial Inhibitors. J. AOAC Int. 81:1135-1140.

Moats, W., et al. 1998. Determination of Free Metabolites of Ceftiofur in Animal Tissues with an Automated Liquid Chromatographic Cleanup. J. AOAC Int. 81:709-713.

National Beef Quality Audit, 2000. (http://www.bqa.org/audits.aspx)

Paige, J. C., L. Tollefson, and M. A. Miller. 1999. Health implications of residues of veterinary drugs and chemicals in animal tissues. Vet. Clin. N. Am. Food Anim. Pract. 15:31-43.

Paturkar, A. M., V. S. Waskar, K. V. Mokal, and R. J. Zende. 2005. Antimicrobial drug residues in meat and their public health significance – a review. Ind. J. Anim. Sci. 75:1103-1111.

Payne M., et al. 2004. Comparison of the use of regulatory assays and high-performance liquid chromatography for detection of residues of ceftiofur sodium metabolites in tissue specimens of culled dairy cattle. Am J. Vet. Res. 65:1730-1733.

Pikkematt, M.G. 2009. Microbial screening methods for detection of antibiotic residues in slaughter animals. Anal. Bioanal. Chem.

Raison-Peyron, N., D. Messaad, J. Bousquet, and P. Demoly. 2001. Anaphylaxis to beef in penicillin-allergic patient. Allergy 56:796-797.

Reeves, P., et al. 2007. Residues of veterinary drugs at injection sites. J. Vet. Pharmacol. Therap. 30:1-17.

Schneider, M. J. and S. J. Lehotay. 2008. A comparison of the FAST, Premi and KIS® test for screening antibiotic residues in beef kidney juice and serum. Anal. Bioanal. Chem. 390:1775-1779.

Schneider, M. J., K. Mastovska, S. J. Lehotay, A. R. Lightfield, B. Kinsellab, and C. E. Shultz. 2009. Comparison of screening methods for antibiotics in beef kidney juice and serum. Anal. Chim. Acta . 637:290-297.

Smith, G.W., J. L. Davis, L. A. Tell, L.A., A. I. Webb, and J. E. Riviere. 2008. Extralabel use of nonsteroidal anti-inflammatory drugs in cattle. J. Am. Vet. Med. Assoc. 232:697-701.

Stead, S., et al. 2004. Meeting maximum residue limits: an improved screening technique for the rapid detection of antimicrobial residues in animal food products. Food Additives and Contaminants. 21:216-221.

USDA-FSIS Notice 39-09. May 5, 2009. Electronic sample (eSAMPLE); Phase I – Reporting in-plant fast antimicrobial screen test (FAST) results.

USDA-FSIS Notice 50-09. July 15, 2009. Using the kidney inhibition swab test (KIS®) test to detect antimicrobial drug residues in cattle in selected establishments.

USDA-FSIS. 2009a. 2008 FSIS National Residue Program Data. United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science; published October 2009.

USDA-FSIS National Residue Program for Cattle. March 2010. Audit Report 24601-08-KC. United States Department of Agriculture, Food Safety and Inspection Service, Off of Inspector General, published March 2010.

USDA-FSIS. 2009b. FSIS National Residue Program Scheduled Sampling Plan. United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science; published October 2009.

Waltner-Toews, D. and S. A. McEwen. 1994. Residues of antibacterial and antiparasitic drugs in foods of animal origin – a risk assessment. Prev. Vet. Med. 20:219-234.