Detection of flunixin in the urine of untreated pigs housed with pigs treated with flunixin meglumine at labeled doses[1]

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ABSTRACT: The objective of this study was to determine the likelihood that swine treated with flunixin meglumine could contaminate their environment, which could cause untreated swine housed in the same pen to ingest or absorb enough drug to be detected in their urine. Currently, any detectable level of flunixin found in the urine of pigs exhibited at livestock shows in Texas can disqualify the exhibitor. We conducted 2 trials in this study. The first, a pilot trial, placed pigs in 2 pens, with each pen housing a pig that did not receive a drug and a treated pig that received 2.2 mg/kg of flunixin intramuscularly. This trial demonstrated that transfer of the drug from treated to untreated pigs housed in close proximity was possible. The second trial was conducted using 10 pens, with a treated and untreated pig in each pen. Each pig receiving treatment was randomly selected and administered 2.2 mg/kg of flunixin intramuscularly; then, urine and plasma were collected from all swine for 10 d. Flunixin was detected at or above the limit of detection of 0.1 ng/mL in the urine of all treated and untreated pigs throughout the 10-d trial. Treated pigs had higher urine levels of flunixin than their untreated pen mates for 4 d post-treatment ($P < 0.0001$), but there was no statistical difference between pen mates during the last 5 d of the trial, making it impossible to differentiate treated from untreated pigs.

Key Words: flunixin, limit of detection, show pigs, treated-untreated pigs, urine levels

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INTRODUCTION

The Food and Drug Administration (FDA) determines acceptable tissue tolerance level for drugs approved for and used in a specific food animal livestock species that enter the human food supply. These restrictions also apply to livestock exhibited by youth at livestock shows. Livestock shows test urine from animals to ensure that residues do not enter the food supply and exhibitors do not receive an unfair competitive advantage through the use of drugs. Although limited published data exist to correlate urine drug levels with levels that might be found in tissues, many livestock shows have developed zero-tolerance drug policies that include potential

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penalties if any level of drug is detected in the urine. Modern technology allows detection of drugs in minute concentrations and finding a drug level greater or equal to the limit of detection (LOD) of 0.1 ng/mL can result in the exhibitor being disqualified.

Flunixin meglumine is a nonsteroidal anti-inflammatory drug (NSAID) approved for control of pyrexia associated with swine respiratory disease. It is also commonly used to control pain and inflammation in pigs and can be used by livestock show exhibitors to mask symptoms of physical ailments. Research reports that horses treated with an NSAID can contaminate their environment allowing untreated horses housed in proximity to test positive during drug testing (Barker, 2008). It is hypothesized that treated and untreated pigs housed in close proximity in a stock show scenario would both have detectable levels of flunixin in their urine. The objective of this study was to test the hypothesis that if treated and untreated pigs were placed in the same pen and urine drug concentrations were measured from treatment until withdrawal, untreated pigs would have detectable levels of flunixin in their urine.

MATERIALS AND METHODS

Animals and Treatments

This study was approved by the Agriculture Animal Care and Use Committee, Texas A&M AgriLife Research (AUP 2017-0348). Two trials were conducted, as described below.

Trial 1 A preliminary trial was performed using 4 Yorkshire gilts obtained from the university swine herd. The gilts (weight range 95.2 to 116.5 kg) were randomly assigned to 2 pens, with each pen containing a treated and untreated animal. The gilt in each pen destined to receive flunixin treatment was selected by a coin toss. One pen was bedded with sorghum straw and the other with commercial pine wood shavings.

Blood and urine were collected on day −3. Blood was collected via venipuncture of the right jugular vein into a 7-mL K3 EDTA tube, centrifuged at 419 × *g* for 15 min, and plasma was placed in plastic tubes and refrigerated until analysis. Urine was collected by placing a tampon in the gilt's vagina, and after urination, the tampon was placed into a 50-mL BD Falcon tube (BD, Franklin Lakes, NJ), and urine was digitally extracted into the tube and immediately refrigerated until analysis. A fresh pair of exam gloves was used when placing the tampons and every attempt was made to collect free flow urine into the 50-mL BD Falcon tube. In the event, the gilt ceased urinate before collection, the tampon was extracted and the urine digitally extracted into the tube using a new pair of examination gloves. On day 1, treated animals received a labeled dose of 2.2 mg/kg of flunixin meglumine (Banamine-S, Merck Animal Health, Madison, NJ) through an 18-gauge 3.81-cm needle intramuscularly (IM) in the neck. Untreated animals were not given a placebo or sham injection. The treated animals were allowed 24 h to potentially contaminate the pen environment, and the bedding was not removed during this time period. Blood for plasma and urine were collected on days 2, 3, 6, and 12, with 12 d being the labeled meat withdrawal time for flunixin in pigs. On day 14, after removing the first gilts, a new untreated gilt was placed in each pen for 4 d, and blood and urine samples were collected as described for the beginning of the trial on days 14, 16, 17, and 18.

Trial 2 In a follow-up to the first trial, 20 Duroc, Hampshire, or Yorkshire gilts (weight range 90.72 to 104.3 kg) were randomly assigned to 10 pens (2 gilts per pen), with a 0.61-m sand barrier between individual pens and a 0.9-m sand alley between groups of pens (Fig. 1). All 10 pens were bedded with commercial pine wood shavings—the most common bedding used at livestock shows. Treatment gilts in each pen were selected using an

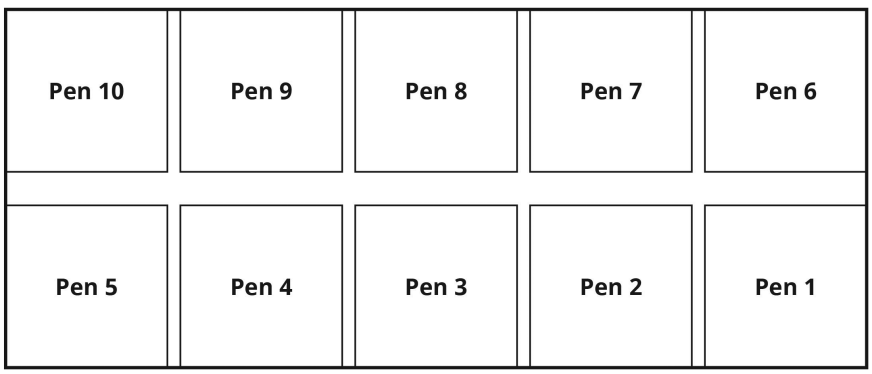

Figure 1. Pen configuration. The 10 pens were 3.05 × 3.05 m with 0.6-m space between pens and a 0.9-m alley.

online random generator. Samples were collected from all gilts on day −3 to ensure that there was no detectable level of flunixin meglumine (>0.1 ng/mL) in urine or plasma. At 0600 h on day 0, the gilts designated for treatment in each pen were administered 2.2 mg/kg of flunixin meglumine IM using an 18-gauge 3.81-cm needle. Untreated gilts were not injected with a placebo or sham injection. Urine was collected from all gilts on days 1, 2, 3, 4, 5, and 9 as described previously in trial 1. Blood for plasma was collected from all gilts on day 1, and plasma was collected from 5 pens on days 2 and 4 and from the other pens on days 3 and 5.

Drug Analysis

All samples were analyzed by the Texas A&M Veterinary Medical Diagnostic Laboratory. Chemicals and reagents included methyl tert-butyl ether (MTBE) and acetonitrile (ACN; LC–MS grade; VWR International, Radnor, PA), formic acid (LC–MS grade; Fisher Scientific, Pittsburgh, PA), and flunixin and flunixin-d3 (Sigma–Aldrich, St. Louis, MO).

Plasma samples were prepared by combining 0.5 mL of plasma with 5-mL MTBE and 100 µL 6N HCl into a screw-top tube. The tubes were rotary racked for approximately 10 min and then centrifuged for approximately 5 min at 419 × *g*. The supernatant was transferred to a second tube and evaporated to dryness under a stream of nitrogen at approximately 45°C. The residue was reconstituted in 80 µL ACN and transferred to an autosampler vial for LC–MS analysis.

Urine samples were prepared by combining 1 mL of urine with 1 mL 0.1 N sodium hydroxide. The tubes were allowed to incubate at room temperature (21°C) for approximately 10 min. Five-milliliter MTBE and 4-mL saturated phosphate buffer (pH 3.2) were added, and the tubes were rotary racked for approximately 10 min and then centrifuged for approximately 5 min at 419 × *g*. The supernatant was transferred to a second tube and evaporated to dryness under a stream of nitrogen at approximately 45°C. The residue was reconstituted in 80 µL 5% ACN and transferred to an autosampler vial for LC–MS analysis.

LC–MS/MS analysis was performed using an Agilent 6400 triple quadrupole mass spectrometer with an electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, CA). Chromatographic separation was performed using an Ascentis Express C18 column (100 × 2.1 mm ID, 2.7 µm) with a C18 guard column (5 × 2.1 mm ID, 2.7 µm; Sigma–Aldrich, St. Louis, MO) maintained at 40°C. The mobile phase consisted of 1) water/formic acid, 100:0.1 v/v, and 2) ACN/formic acid, 100:0.1 v/v. Separation was achieved using a gradient flow rate (0.5 mL/min). Data were collected in positive ion mode by multiple reaction monitoring of the transition *m*/*z* 297.1 to *m*/*z* 279.0 and *m*/*z* 264.1 for flunixin, and *m*/*z* 300.1 to *m*/*z* 264.1 and *m*/*z* 140.1 for flunixin-d3. The optimized parameter settings for ESI included a capillary voltage of 4.0 kV, gas temperature of 350°C, gas flow of 10 L/min, and nebulizer of 50 psi. The injection volume was 10 µL. The LOD for urine and serum was 0.1 ng/mL, and the limit of quantitation for urine and serum was determined to be 0.2 ng/mL. Method validation was performed by analyzing multiple samples at fortified concentrations and the accuracy and precision of the assay were determined to be 100.4% and 96.1% for serum, respectively, and 100.3% and 97.9% for urine, respectively. Quantitation of flunixin was performed using a concurrently analyzed calibration curve.

Statistical Analysis

The pen was the experimental unit with 1 treated gilt and 1 untreated gilt in each pen. Flunixin meglumine concentrations were log-transformed prior to analysis using natural logarithm for analysis. Analyses were performed with PROC MIXED (SAS Institute Inc., Cary, NC) using repeated measures design (days) in which animals were the subjects and an autoregressive covariance structure was assumed. The covariance structure was selected based on minimization of the Bayesian information criterion. Treatment, day, and animal interaction were included as fixed effects. When sufficient interaction effects were observed, treatment means were compared within the day to resolve effects. Because 0.1 ng/mL is considered the LOD for livestock show sample screening, in the laboratory used for analysis, it was used as the cutoff for positive results in this experiment.

RESULTS

During the first trial, urine flunixin concentrations remained greater than 0.1 ng/mL through day 12 in all treated and untreated gilts demonstrating that cross-contamination did occur (Table 1). The 4 gilts were removed on day 12, and 2 untreated gilts (one in each pen) were introduced to determine whether untreated animals could absorb or ingest a sufficient amount of drug that would be detected in the urine. Interestingly, these pigs' urine contained flunixin concentrations of 15 and 0.9 ng/mL prior

to being placed in the pens (see Table 1). These gilts came from a pen of feeder pigs and treatment records did not indicate the pigs in question had ever received flunixin; however, other animals in the pen had been treated with the drug.

The second trial involved placing 20 gilts—as pairs—in 10 pens. Each pen housed 1 treated gilt and 1 untreated gilt. The 20 gilts in this phase had no detectable levels of flunixin meglumine in urine or plasma on day −3. During the first 4 d post-treatment, the treated gilts had higher levels of flunixin in their urine than the untreated gilts ($P > 0.0001$). On days 4, 5, and 9, no significant differences in urine drug values existed between treated and untreated gilts; however, all 20 gilts enrolled in this experiment had ≥0.1 ng/mL of flunixin meglumine on day 13, the conclusion of the experiment. Table 2 displays the findings. Results from this study indicate pigs treated with the labeled dose of flunixin and housed with untreated pigs can result in untreated pigs having detectable drug levels in their urine.

DISCUSSION

We believe this is the first study to investigate the risk associated with housing pigs treated with

Table 1. Urine and plasma collection schedule for trial 1

	Urine levels, ng/mL					
Date	Pig 45[1]	Pig 50[2]	Pig 47[1]	Pig 49[2]	Pig 33N[3]	Pig 49N[3]
Day −3	0	0.1	0	0.1		
Day 2	179	54	143	1.2		
Day 3	1.9	6.3	32	0.6		
Day 6	0.2	0.1	10	0.1		
Day 12	18	0.1	0.1	0.3		
Day 14					0.9	15
Day 16					27	5.8
Day 17					2.6	0.9
Day 18					3.7	1.1

[1]Treated gilts.

[2]Untreated gilts.

[3]Nontreated animals used to access the likelihood of environmental contamination.

Table 2. Urine collection times and results for the 10 pens in phase 2[1]

		Weight	Bled	Urine	Treatment	Urine	Urine	Urine	Urine	Urine	Urine
Pen	Pig	Day −3	Day −3	Day −3	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 9
1	1	201	0	0	4 mL	2,811	187.5	6.6	2.8	1	2.5
1	2	200	0	0		1	4.7	5.4	4.2	3.1	4
2	3	205	0	0	4 mL	6,164.8	79.4	19.6	9.6	1.1	1.4
2	4	215	0	0		1.7	1.7	2.9	1.4	0.9	0.5
3	5	202	0	0		2	2	1.4	0.9	1.4	1.2
3	6	215	0	0	4.2 mL	1,676.1	278.9	53.4	5	3.1	2.8
4	7	225	0	0	4.4 mL	2,044.9	80.1	9.4	2.1	1.6	2.2
4	8	219	0	0		2.8	3.1	11.2	2.5	3	0.2
5	9	209	0	0		4.7	135	4	5.8	1.7	0.1
5	10	209	0	0	4 mL	474	17.6	5.9	1.3	0.8	1.3
6	11	218	0	0	4.2 mL	2,040.2	161.3	26.7	3.3	1.2	1.1
6	12	199	0	0		2.6	1.9	1.3	2.7	0.5	1.4
7	13	219	0	0	4.2 mL	2,485.2	92.1	4.6	0.7	21.6	1.1
7	14	210	0	0		3.4	2.6	3.4	7.3	2.7	1.9
8	15	230	0	0	4.6 mL	1,758.8	180.2	37.6	16.7	7.6	0.6
8	16	207	0	0		2.1	4.2	3.3	3	1.3	1
9	17	202	0	0		7.4	10.5	6.2	0.7	3.3	0.7
9	18	232	0	0	4.6 mL	2,724.2	161.2	33.9	1.4	5.2	2.4
10	19	204	0	0	4 mL	1,316.5	355.8	53.9	22.9	10.7	6.2
10	20	221	0	0		10.4	23.9	22	8.6	3.2	1

[1]Raw data urine (ng/mL).

flunixin in close proximity to untreated pigs in a livestock show scenario. Coetzee (2015) worked with finishing-age swine in a commercial production and demonstrated that drugs excreted in urine and oral fluids of treated swine served as a source of drug contamination to untreated animals. Investigators in the United Kingdom and France have demonstrated the ability of horses treated with NSAIDs to recycle the drug through contaminated bedding, causing untreated pen mates to test positive (Norgren et al., 2000; Popot et al., 2007, 2011). Sheep treated with flunixin have been shown to concentrate the drug in their wool; thus, wool biting could elicit a positive drug test in untreated herd mates (Richards et al., 2011). Performance-enhancing drugs have been found in the environment of an equine race track, including locations such as the testing barn, stalls, and lagoons holding runoff water (Barker, 2008).

Youth participating in managing and exhibiting livestock have the same responsibilities as commercial livestock producers in caring for their animals and ensuring a safe food supply. They refine their knowledge in animal husbandry to include principles such as genetic selection, basic nutrition, health, animal welfare, and food safety aspects to include proper use of drugs to ensure no violative drug residues enter the food supply. Pain management in food animal medicine has come of age, with livestock owners and veterinarians considering pain mitigation for routine surgical procedures such as castration and management of pain associated with injuries and disease (Fajt et al., 2011). Therapeutic drugs used for mitigation of pain can mask lameness due to disease, injury, or genetic predisposition; therefore, livestock shows have adopted a zero-tolerance policy for any drug detected in the urine of animals being exhibited to ensure a level playing field. Livestock shows and adult advisors assist with the educational process, and they have additional responsibilities to ensure performance-enhancing drugs are not tolerated.

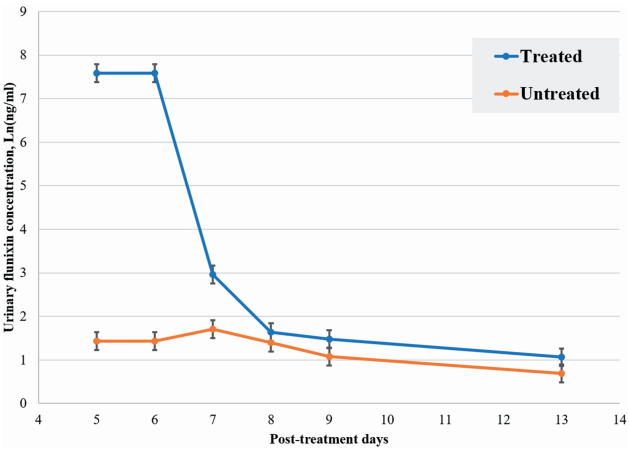

Figure 2. Urine concentration in treated and untreated swine. Treatment* day interaction was significant at $P < 0.001$. Treatment was greater than control on days 5, 6, and 7 ($P < 0.0001$), but not on day 8 ($P = 0.40$), day 9 ($P = 0.17$), or day 13 ($P = 0.21$). Ln is the natural logarithm of the flunixin concentration (ng/mL) in the urine.

In our study, the gilts receiving flunixin had significantly higher urine drug levels than the gilts that did not receive flunixin on days 5, 6, and 7; however, on days 8 through 13, all 20 gilts continued to test positive, with no difference between treated and untreated, making it impossible to distinguish the treated animals versus untreated animals (Fig. 2). Through the course of this experiment, all treated and untreated gilts' urine consistently contained ≥0.1 ng/mL of flunixin. The LOD reported by the laboratory is 0.1 ng/mL, and currently, ≥0.1 ng/mL of flunixin in the urine can disqualify exhibitors whose animals are housed in close proximity to a flunixin-treated animal. The number of treated and untreated animals in this study that would be disqualified using 0.1, 5, 10, 20, and 30 µg/mL values of urine flunixin is shown in Table 3. This study did not determine how untreated pigs became exposed to the drug, but we speculate that contamination occurred from the commercial pine wood shavings used for bedding. A previous study has shown the drug levels in the

Table 3. The number of treated and untreated pigs that would be disqualified using maximum concentrations of 0.1, 5, 10, 20, and 30 ng/mL

		Maximum allowable concentration, ng/mL				
Date	Group	0.1	5	10	20	30
Day 1	Treated	10	10	10	10	10
	Untreated	10	2	1	0	0
Day 3	Treated	10	9	6	5	4
	Untreated	10	4	2	1	0
Day 5	Treated	10	4	2	1	0
	Untreated	10	0	0	0	0
Day 9	Treated	10	1	0	0	0
	Untreated	10	0	0	0	0

urine and oral fluids of untreated swine (Coetzee, 2015). The risks of possible exposure of untreated pigs to drugs during transport and exhibition need to be evaluated, and appropriate biosecurity measures must be taken to mitigate those risks. Veterinarians must be aware of the concept of environmental contamination when treating or prescribing drugs for animals exhibited at livestock shows. It is imperative that all drugs be administered according to label, and if drugs are being used in an extra-label manner, a reasonable extended withdrawal time must be applied. Drugs used in an unapproved species may be allowed under the conditions of the Animal Medical Drug Clarification Act of 1994 (AMDUCA); however, a drug tolerance is not allowed under those circumstances (FDA, 1994).

CONCLUSION

The results of our study suggest that swine treated with flunixin can recycle the drug in their environment, and untreated pigs can ingest adequate drug amounts from the environment to elicit a positive drug test. The study demonstrates the need for flexibility when assessing quantitative laboratory information concerning drugs detected in livestock urine. More research confirming the relationship of urine and residue levels with performance enhancement is needed. The zero-tolerance policy is appropriate for drugs used in an extra-label fashion in species without an approved label; however, extremely low levels of any extra-label drugs should be interpreted with caution because of the possibility of environmental contamination.

County extension agents, agricultural science teachers, parents, and veterinarians must understand this concept and manage accordingly when transporting or confining animals. Drugs administered to food animals must be based on sound diagnostics with strict adherence to medication labels. In the event of extra-label use, all restrictions set forth in AMDUCA must be observed (FDA, 1994). Concise treatment records are essential and must include the condition diagnosed, and for all drugs administered, the dose, withdrawal date, and route of administration are recorded.

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