PEER REVIEWED

Clinical effectiveness of enrofloxacin 100 mg/mL injectable solution for the treatment of acute anaplasmosis in cattle caused by *Anaplasma marginale*

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Abstract

Anaplasma marginale is a gram-negative rickettsial pathogen that can cause clinical anemia and death in cattle. The objective of this study was to evaluate the effectiveness of enrofloxacin (ENR) 100 mg/mL at a single subcutaneous dose of 5.7 mg/lb (12.5 mg/kg) for treatment of acute anaplasmosis (ANA) in mature beef cows (n=67). Following intravenous inoculation with *A. marginale*-infected blood, cattle were monitored for clinical signs of ANA. Upon meeting case criteria, cattle were randomly assigned to receive ENR or saline (SAL). Treatment success, defined as 28 d post-treatment survival and resolution of abnormal clinical scores, was 81.8% (27/33) and 44.1% (15/34) (P=0.0032) for ENR and SAL treated cows, respectively. Mortality was 47% (16/34) and 3% (1/33) in SAL and ENR, respectively (P=0.0027). Packed cell volume at 7, 14, 21, and 28 d post-treatment was significantly greater in ENR compared to SAL (P<0.05). In this study, ENR improved treatment success compared to SAL, reduced ANA mortality, and maintained greater packed cell volumes post-clinical signs compared to SAL. Extra-label use of fluoroquinolones in food animals is prohibited in the United States, but ENR (Baytril® 100-CA1) was recently conditionally approved for treatment of ANA.

Key words: *Anaplasma marginale*, anaplasmosis, treatment, enrofloxacin, bovine

Résumé

Anaplasma marginale est une espèce de rickettsie pathogène gram-négative qui peut causer l'anémie clinique et la mort chez les bovins. L'objectif de cette étude était d'évaluer l'efficacité de l'enrofloxacine (ENR) 100 mg/ml en dose sous-cutanée unique à 5.7 mg/lb (12.5 mg/kg) pour le traitement de l'anaplasmose aigüe (ANA) chez des vaches de boucherie adultes (n=67). Suivant l'inoculation intraveineuse avec du sang infecté avec *A. marginale*, les signes cliniques de l'ANA ont été surveillés chez les bovins. Les bovins rencontrant les critères d'inclusion pour un cas ont été alloués au hasard dans un groupe recevant soit l'ENR ou soit la saline (SAL). Le taux de succès du traitement, défini comme étant la survie 28 jours après le traitement avec résolution des scores cliniques anormaux, était de 81.8% (27/33) chez les vaches du traitement ENR et de 44.1% (15/34) chez les vaches du traitement SAL (P=0.0032). Le taux de mortalité était de 47% (16/34) chez les vaches SAL et de 3% (1/33) chez les vaches ENR (P=0.0027). L'hématocrite aux jours 7, 14, 21 et 28 suivant le traitement était significativement plus élevé dans le traitement ENR que dans le traitement SAL (P<0.05). Dans cette étude, l'ENR a augmenté le taux de succès par rapport au traitement SAL, a diminué le taux de mortalité relié à l'ANA et maintenu l'hématocrite a de plus grandes valeurs suivant le traitement que SAL. L'utilisation des fluoroquinolones en dérogation des directives de l'étiquette n'est pas permise aux États-Unis mais l'ENR (Baytril® 100-CA1) a reçu récemment une approbation conditionnelle pour le traitement de l'ANA.

Introduction

Anaplasma marginale is a gram-negative blood-borne rickettsia that has been identified in all 48 contiguous states within the United States and other regions across the world.[14] The organism parasitizes host erythrocytes and often results

in fever, lethargy, increased respiratory rate, severe anemia, decreased milk production, and death in acute cases, especially in cattle greater than 2 years of age.[1,16] Recovery is common in younger animals, but mortality in mature animals presenting with clinical signs may be 50 to 60%. Pathogen load and anemia rapidly progress once clinical signs manifest themselves. Thus, intervening while there are enough erythrocytes for the animal to survive is critical. The conventional treatment for acute clinical anaplasmosis (ANA) is parenteral administration of a long-acting oxytetracycline, but this antimicrobial has a bacteriostatic, time-dependent pharmacodynamic profile. Therefore, successful therapy of ANA with oxytetracycline takes time and requires a competent host immune response to work in conjunction with the antimicrobial to control the infection.[6] Imidocarb has been shown to be an effective antimicrobial intervention, but the product is not registered in the United States for use in cattle, and global use is restricted due to safety concerns.[8,9]

Fluoroquinolone antimicrobials, such as enrofloxacin (ENR), are considered effective for the treatment of ANA.[14] Extra-label use of fluoroquinolones in the United States is prohibited, which has likely limited the evaluation of fluoroquinolone antimicrobials for treatment of ANA. Past research demonstrated that enrofloxacin is effective at killing *A. marginale in vitro*.[6,7] Other identified research provides evidence of ENR effectiveness for treatment of ANA.[2,3,5,10,12,15,17,18] In Brazil, an ENR 100 mg/mL product has a label indication for the treatment of ANA at a single, subcutaneous (SC) dosage of 3.4 mg/lb (7.5 mg/kg) of body weight (BW).[a] It has also been reported that an ENR dose of 5.7 mL/100 lb (5.7 mg/lb; 12.5 mg/kg) SC twice 48 h apart is effective for treatment of ANA.[5] A single SC dose of 5.7 mL/100 lb (5.7 mg/lb; 12.5 mg/kg) was selected to investigate on the basis of available literature, clinical experience, and the desire to use existing ENR dosages that result in the greatest maximum concentrations. This is especially true when considering most animals treated for ANA are mature cattle which may be pregnant or lactating, and have a higher metabolic rate. Despite available research, effectiveness of ENR for the treatment of ANA using a dose of 5.7 mL/100 lb of BW (5.7 mg/lb; 12.5 mg/kg of BW) administered once by SC injection has not been fully characterized, and warrants further investigation. Thus, the objective of the study presented here was to demonstrate the effectiveness of a single treatment with enrofloxacin (100 mg/mL) at 5.7 mL/100 lb of BW (5.7 mg/lb; 12.5 mg/kg of BW) for treatment of ANA in randomized and masked clinical trials.

Material and Methods

The study was approved by the Midwest Veterinary Services, Inc. Institutional Animal Care and Use Committee under ethical approval number AC18021B. The study was performed in accordance with current standard operating procedures and the Bayer Animal Health Animal Welfare Policy. All aspects of the study met or exceeded recommendations described in the National Institutes of Health guide for the care and use of laboratory animals.

Study Animals

The study was initiated March 15, 2018. Animals were Angus beef cows at least 2 years of age (n=73), and weighed an average 1543 lb (700 kg; range 1243 to 1885 lb [564 to 855 kg]). Cows were provided an acclimation period of 30 d prior to inclusion in the study. Cows originated from a cow-calf herd in South Dakota and were confirmed to be negative for *A. marginale* via cELISA, reverse transcriptase-PCR, and blood smear evaluation. The cELISA and reverse transcriptase-PCR assays were performed by the Kansas State Veterinary Diagnostic Laboratory, and blood smear evaluations were performed by the Central States Research Center Supporting Laboratory. Cows were housed in a standard outdoor dry-lot pen, and were provided ad libitum feed and water. Treatments were commingled within the pen.

Inoculum characterization and collection

A cow naturally infected with *A. marginale* was identified and used as the inoculum donor. The donor cow originated from a cow-calf herd in Oklahoma that had experienced an *A. marginale* outbreak in 2017, although the cow was procured from a herd in Southeast Kansas in early 2018. This donor cow was determined to be infected with the *A. marginale* Msp1a genotype M-F-F. The donor cow was properly restrained and 55 mL of infected blood at a time was collected from the jugular vein using a 16-gauge 1.5 in (3.8 cm) needle attached to a 60 mL pre-heparinized syringe. This was performed multiple times to collect sufficient volume to inoculate every animal with 55 mL of infected blood. The inoculum was immediately stored on ice until inoculation of study animals, which occurred within 12 h of sample collection.

A standard volumetric challenge dose of 55 mL was selected for this study on the basis that it results in clinical disease regardless of bacteremia and stage of ANA of the donor. In the experience and opinion of the authors, the volumetric challenge dose of 55 mL is known to reliably produce ANA in animals.[b] The inoculum was evaluated to determine the percent parasitized erythrocytes (PPE) by preparing a thin blood smear in order to determine potential bacterial load in the inoculum. A total of 500 erythrocytes were counted, and the number of infected cells divided by 500 and then multiplied by 100 to obtain the PPE. The PPE of the inoculum was determined to be 41.2%.

Inoculation procedure

Prior to inoculation, a blood sample was obtained for packed cell volume (PCV) determination. Study animals were properly restrained and blood was collected from the jugular vein using a 14-gauge 1.5 in (3.8 cm) needle attached to a 2 mL CA-EDTA vacutainer tube.

Animals were similarly handled for inoculation, except

that a 16-gauge 1.5 in (3.8 cm) needle was attached to a 60 mL syringe filled with the 55 mL of heparinized *A. marginale* infected blood from the donor cow was advanced into the vein. The plunger of the syringe was withdrawn to allow the remaining 5 mL of the syringe to fill with the recipient's blood. The entire 60 mL admixture of inoculum and the individual recipient's own venous blood was then slowly injected into the vein. A total of 73 cows were inoculated.

Post-inoculation clinical monitoring

Inoculated animals were monitored twice daily for evidence of clinical disease (i.e., depression score, mucous membrane score, rectal temperature) from the day following inoculation until the time of treatment using pre-specified scoring systems (Table 1). Animals exhibiting a depression score of 2 or greater were removed from the pen to obtain a mucous membrane score and rectal temperature. The case definition of ANA was:

Depression score ≥ 2 and mucous membrane score ≥ 1 and rectal temperature ≥ 104.0°F (40.0°C).

Experimental design and treatments

On the day study animals met ANA criteria they were randomly assigned to 1 of 2 treatment groups by an individual not masked to treatment:

1. ENR: enrofloxacin[c] 100 mg/mL at a dose rate of 5.7 mL/100 lb of BW (12.5 mg/kg of BW) administered SC once.
2. SAL: 0.9% saline[d] given SC once at an equivalent volume to ENR.

A blood sample was collected at the time of treatment for PCV determination and blood smear evaluation for presence of *A. marginale* organisms. The animals were assessed twice daily for general health following treatment, and were only assigned clinical scores at the time of clinical assessment described in the next section.

Cows with ANA were moved to a new dry-lot pen following experimental treatment with ENR and SAL to separate treated animals from those that had not met case criteria up to that point in time. Animals from each treatment group were commingled within the same pen.

Clinical assessments and sample collection

Treated animals were assessed for treatment success/failure once every 7 d until study d 28 by individuals masked to treatment. Treatment success/failure was assigned for each time point and was not considered a summary measure, meaning that an animal could be classified as a treatment failure at 1 time point and a treatment success at another (assuming survival). A blood sample for PCV determination was collected at each clinical assessment. A treatment failure was considered to be:

Depression score ≥ 2 or mucous membrane score ≥ 1 or rectal temperature ≥ 104.0°F (40.0°C).

Animals that died or were humanely euthanized due to severe disease following treatment were also considered treatment failures. Animals not classified as treatment failures were considered treatment successes.

Analytical procedures

PCV determination

Packed cell volumes were determined by filling a capillary tube approximately ¾ full from a blood sample in a CA-EDTA blood tube and sealing 1 end of the tube with clay.[e] Capillary tubes were then centrifuged for 5 min at 13,000 x *g*.[f] The capillary tube was read using a standard hematocrit reader by an individual masked to treatment. Results were recorded on pre-designed forms. Packed cell volumes were determined immediately prior to inoculation, at the time of treatment, and again at 7, 14, 21, and 28 d post-treatment.

Blood smear evaluation

Thin blood smears were prepared by pipetting 50 µL of blood collected at the time of treatment in CA-EDTA tubes onto a clean slide. A second slide was then held at a 30 to 40 degree angle on the first slide, and was drawn backwards to allow an even spread of blood on the slide. Slides were stained using a standard Giemsa staining procedure.[g] Blood smears were evaluated by a trained veterinarian masked to treatment. Presence or absence of *A. marginale* organisms was recorded on a pre-designed form.

Table 1. Clinical measurement systems used in the present study to assess the development of acute clinical anaplasmosis in cattle caused by *Anaplasma marginale* following intravenous inoculation with 55 mL of heparinized blood from a cow naturally infected with *Anaplasma marginale*.

Scoring Systems	
Depression Score	**Mucous Membrane Score**
0 = normal: alert, active, normal appetite, well-hydrated, coat normal	0 = normal: mucous membranes have normal color with no paleness or icterus
1 = mild: moves slower than normal, slightly rough coat, may appear lethargic but upon stimulation appears normal	1 = pale: mucous membranes are pale in appearance
2 = moderate: inactive, may be recumbent but is able to stand, gaunt, may be dehydrated	2 = icteric: mucous membranes are icteric (yellow) in appearance
3 = severe: down or reluctant to get up, gauntness evident, dehydrated	

Necropsy

Gross necropsies were performed on animals that died or were euthanized due to severe ANA in order to confirm gross pathologic findings consistent with ANA.

Study termination

Cows that completed the study were returned to commerce. ENR cows were not returned to commerce sooner than 28 d following experimental treatment. This was done in compliance with an investigational withdrawal period prescribed by Food Use Authorization D-0248 granted by the Food and Drug Administration Center for Veterinary Medicine.

Statistical analysis

Data were double entered into an electronic spreadsheet in a commercial software program with 100% quality control of the data.[h] Outcomes included treatment success percent, mortality percent, and change in PCV. All statistical analyses were performed with commercial statistical software.[i]

The experimental unit was the individual animal. Treatment success and mortality outcomes were each analyzed using the GLIMMIX procedure. Treatment group served as a fixed effect. Treatment success at each clinical assessment was considered to be an independent event and was analyzed separately. For PCV outcomes, the MIXED model procedure was applied having fixed effects for treatment, study day, and the study day by treatment interaction. A random effect for animal id was included in all statistical models, and a p-value of 0.05 or less was used as the significance level.

Results

Post-inoculation monitoring and enrollment

Of the 73 inoculated cows, a total of 67 met case criteria and were assigned to treatment and retained in the analysis (ENR n=33, SAL n=34). The remaining 6 cows either died due to severe ANA prior to receiving treatment (n=2), did not meet ANA case criteria when treatment was given (n=1), did not meet ANA criteria during the post-inoculation monitoring period (n = 1), or were removed from the study prior to meeting ANA case criteria for infectious pododermatitis that was treated with tulathromycin[j] 100 mg/mL at a single SC dose of 1.1 mL/100 lb of BW (1.13 mg/lb; 2.5 mg/kg BW; n=2).

Cows met the ANA criteria 25 to 28 d post-inoculation, and also presented with tachypnea and a grimaced facial expression (Figure 1).

Blood smear evaluation

All cows were found to have *A. marginale* organisms present on blood smear on the day ANA case criteria were met. Based on the results, all cows were confirmed to be positive for *A. marginale* at the time of treatment.

Figure 1. Beef cows with acute clinical anaplasmosis 25 to 28 days post-inoculation with 55 mL of *Anplasma marginale* infected blood. Cows were exhibiting a grimace expression in addition to meeting clinical case criteria to be eligible for treatment.

Table 2. Treatment success outcomes at 7, 14, 21, and 28 days post-treatment for female beef cattle ≥ 2 years of age and inoculated with *Anplasma marginale*. Animals were treated once clinical anaplasmosis case criteria were met with enrofloxacin 100 mg/mL at a single subcutaneous dose of 5.7 mg/lb (12.5 mg/kg) (ENR) or with 0.9% saline at an equivalent volume to ENR (SAL). Case criteria were met when cows demonstrated a depression score ≥ 2, a mucous membrane score ≥ 1, and a rectal temperature ≥ 104.0°F (40.0°C). Treatment failures were defined as having a depression score ≥ 2 or mucous membrane score ≥ 1 or rectal temperature ≥ 104.0°F (40.0°C). Animals that died or were humanely euthanized due to severe disease following treatment were also considered treatment failures.

Study Day	Treatment Group*	N	Treatment Success (%)	*P*-value
7	ENR	33	24 (72.7%)	0.0130
	SAL	34	14 (41.2%)	
14	ENR	33	23 (69.7%)	0.0075
	SAL	34	12 (35.3%)	
21	ENR	33	26 (78.8%)	0.0035
	SAL	34	14 (41.2%)	
28	ENR	33	27 (81.8%)	0.0032
	SAL	34	15 (44.1%)	

*ENR = enrofloxacin 100 mg/mL at 5.7 mg/lb (12.5 mg/kg) given subcutaneously once, SAL = saline control given at equivalent volume to ENR

Clinical assessments

The treatment success percent in ENR-treated cows was significantly higher than in SAL-treated cows at each clinical assessment ($P \leq 0.05$; Table 2).

Mortality

Risk of mortality (death and humane euthanasia) due to severe anaplasmosis was significantly greater in SAL-treated cattle compared to ENR-treated cattle (p=0.0027). Mortality due to severe ANA in SAL-treated cattle was 47% (n=16), and 3% (n=1) in ENR-treated cattle.

Death or euthanasia due to severe ANA in ENR and SAL-treated cows occurred within 7 d of receiving treatment. Gross necropsy findings were consistent with ANA, and included pale or icteric mucous membranes, splenomegaly, and watery blood. Heart blood samples were collected from all animals that died except from 1 SAL treated cow. The PCV of heart blood samples ranged from 0 to 13%, but was not analyzed statistically. All heart blood samples were observed to have *A. marginale* identified via blood smear. All gross findings were consistent with ANA, therefore no further diagnostics were performed.

PCV

Packed cell volume at d 7, d 14, d 21, and d 28 post-treatment was significantly greater in ENR-treated cows than SAL-treated cows ($p \leq 0.05$) (Figure 2). Cattle experienced a decrease in PCV of approximately 12% between inoculation and treatment. ENR-treated cows had a 2.3% PCV decrease between treatment and d 7 post-treatment compared to a 13.3% PCV decrease in SAL-treated cattle at the same time points. Surviving ENR and SAL-treated cows returned to near pre-inoculation PCV levels by d 28 post-treatment.

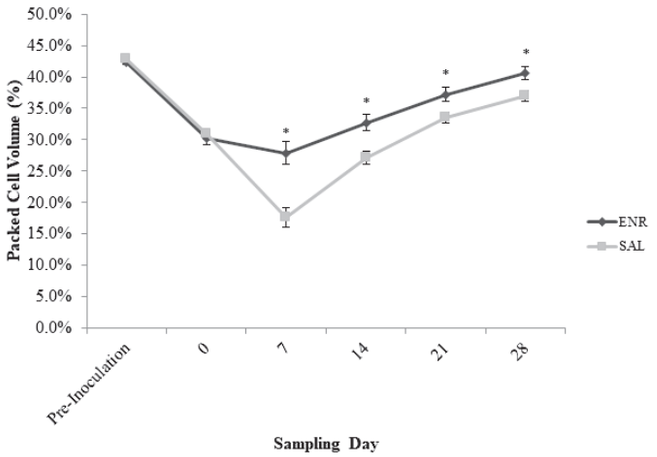

Figure 2. Packed cell volumes for beef cows inoculated with *Anaplasma marginale* subsequently treated for acute clinical anaplasmosis. Treatment day was d 0. ENR = enrofloxacin 100 mg/mL at 5.7 mg/lb (12.5 mg/kg) given subcutaneously once, SAL = saline control given at equivalent volume to ENR. *indicates statistically significant difference between treatment groups ($P < 0.05$).

Discussion

Extra-label use of fluoroquinolones in the United States is prohibited. This study was performed using an enrofloxacin 100 mg/mL product approved for the treatment of bovine respiratory disease[c] under experimental authorization from the Food and Drug Administration Center for Veterinary Medicine. A conditionally approved enrofloxacin 100 mg/mL product[k] for the treatment of clinical anaplasmosis has recently become available in the United States and may be used to treat ANA. Veterinarians should only prescribe products to treat ANA in cattle that are labelled for the treatment of ANA in cattle, and currently only 1 enrofloxacin product[k] is approved to treat this disease. Veterinarians and producers outside of the United States should consult local laws, policies, and products to determine the most effective legal treatment options available for ANA.

The objective of this study was to evaluate the effectiveness of ENR for treatment of ANA at a dose of 5.7 mL/100 lb of BW (5.7 mg/lb; 12.5 mg/kg of BW) administered once SC. This study represents the first publicly reported randomized clinical study evaluating a single 5.7 mL/100 lb of BW (5.7 mg/lb; 12.5 mg/kg of BW) SC dose of ENR in mature cattle with ANA known to the authors. Treatment eligibility in this study was solely based on clinical case criteria as opposed to using diagnostic criteria or pathogen load as has been done in the published literature. Important to note is that all animals met the following criteria:

1. Experimentally challenged with 55 mL of *A. marginale* infected blood;
2. Met clinical case criteria after an expected incubation period;
3. Were confirmed to have *A. marginale* organisms present via blood smear on the day case criteria were met and animals were treated. Direct observation of the organism concurrent to the time of clinical signs of disease and anemia is the hallmark of diagnosing anaplasmosis. It is known that readily observing the pathogen in a blood smear indicates a PPE >1% and a bacteremia of at least $1x10^7$ *A. marginale*/mL blood.
4. Died or were euthanized within days of meeting case criteria;
5. Gross necropsy findings were consistent with ANA.

This study helps confirm that 55 mL of infectious blood is a reliable volumetric challenge dose. Further, these studies show additional diagnostic evaluation at diagnosis/necropsy would not be expected to provide additional benefit when evaluating the efficacy of an antimicrobial intervention for acute ANA and that a clinical-based approach is appropriate. Results of this study indicate that ENR improves treatment success and mortality outcomes in cattle with ANA compared to SAL controls.

This study demonstrates that enrofloxacin may help prevent death due to ANA when given at the onset of clinical signs based on the statistically significant mortality reduction. Only 1 death occurred in the ENR-treated group compared to 16 deaths in the SAL-treated group (3% and 47%, respectively).

Cows in both treatment groups had a reduction in PCV between inoculation and treatment. Treatment with ENR diminished additional declines in PCV post-treatment observed at d 7 post-treatment compared to SAL-treated cows. The PCV in both groups was observed to increase at d 14 post-treatment relative to d 7 post-treatment. The regenerative response observed in these cows aligns with the onset of peak erythrocyte regeneration of 4 to 7 d post-insult, with an expected restoration to baseline PCV in 2 to 4 weeks.[13] Based on the results of this study, survivors were expected to return to a clinically normal state by d 28 post-treatment based on PCV levels and clinical assessments.

Of interest to the authors was the manifestation of the grimace expression demonstrated in the cows. The grimace expressions were not quantified, but were an observation of the researchers involved with the study. A grimace expression has been also been described as a "pain face" and may have a potential role in identifying pain or discomfort in cattle.[11] To the authors' knowledge this is the first report of a grimace expression being observed in cattle with ANA.

Of greater importance is that a contemporaneous isolate was utilized for the study. Interestingly, the M-F-F genotype from the inoculum has not been reported in the literature in the United States, but this genotype has been identified in other cattle in Kansas and Oklahoma.[b] This isolate potentially exhibits a different virulence and susceptibility profile compared to other isolates, and thus additional studies with other isolates are warranted.

This study utilized an inoculation model of the disease which may not perfectly reflect the naturally occurring field cases. Ideally, a study would be performed in clinical field cases, but is not technically feasible due to the sporadic nature of clinical cases in the field.

Conclusions

This study demonstrated that enrofloxacin administered SC at a dose of 5.7 mL/100 lb of BW (5.7 mg/lb; 12.5 mg/kg of BW) improved treatment success and mortality outcomes when administered to cows with clinical anaplasmosis. Mortality was reduced from 47% to 3% ($p = 0.0027$). Veterinarians and producers should remain aware that extra-label use of fluoroquinolones in food animals is illegal in the United States, but a conditionally approved product containing enrofloxacin 100 mg/mL has recently become available for treatment of anaplasmosis.

Endnotes

[a] Kinetomax®, Bayer S.A., São Paulo, Brazil
[b] Dr. Kathryn Reif, unpublished data
[c] Baytril® 100, Bayer Healthcare LLC, Shawnee, KS
[d] Saline Solution 0.9%, VetOne®, Grand Island, NE
[e] PATI, Globe Scientific Inc. Mahwah, NJ
[f] Autocrit Ultra 3, Becton Dickinson, NJ
[g] Remel, Lenexa, KS
[h] Microsoft Excel version 2010, Microsoft, Redmond, WA
[i] SAS® 9.4, SAS Institute, Inc, Cary, NC
[j] Draxxin®, Zoetis Inc., Kalamazoo, MI
[k] Baytril 100-CA1, Bayer Healthcare LLC, Shawnee, KS

Conflict of Interest

This work was funded by Bayer U.S. LLC, Animal Health. Douglas D. Shane, Ronald K. Tessman, and Yingying Wang were employees of Bayer U.S. LLC, Animal Health. All authors assisted with the design and conduct of the study, interpretation of the data, and manuscript review. There were no conflicting interests that could have influenced the conduct and reporting of this study.

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