

The effects of danofloxacin and tilmicosin on neutrophil function and lung consolidation in beef heifer calves with induced *Pasteurella (Mannheimia) haemolytica* pneumonia

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Pneumonia caused by *Pasteurella (Mannheimia) haemolytica* was induced in weaned beef heifer calves, approximately 6 months of age. Calves were treated at 20 h after challenge with therapeutic doses of danofloxacin or tilmicosin. Peripheral blood neutrophils were collected at 3, 24 and 48 h after treatment. The *ex vivo* effects on neutrophil function, neutrophil apoptosis, and hematological parameters were examined, as was the effect on percentage lung consolidation. Neutrophil function assays included random migration under agarose, cytochrome C reduction, iodination, *Staphylococcus aureus* ingestion, chemotaxis, and antibody-dependent and antibody-independent cell-mediated cytotoxicity. Apoptosis was determined using a cell death detection kit. Killing was performed at 72 h after treatment. Statistical comparisons were made among the three groups of challenged–treated animals: saline, danofloxacin, and tilmicosin. Comparisons were also made between nonchallenged nontreated animals (NCH) and challenged saline-treated animals. There were no significant differences for any of the neutrophil function assays or neutrophil apoptosis among the challenged–treated groups. This suggests that danofloxacin and tilmicosin have no clinically significant effects on neutrophil function or apoptosis. There were also no significant differences in percentage lung consolidation among the challenged–treated groups. Significant differences were found between the NCH calves and the challenged saline-treated calves in several neutrophil assays, which were attributed to effects of *P. haemolytica* infection.

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INTRODUCTION

The effect of antimicrobials on immune function is a burgeoning area of investigation in veterinary medicine. The authors have previously reported that no significant differences were found between two antimicrobials, danofloxacin and tilmicosin, on circulating neutrophil function in healthy beef calves (Fajt *et al.*, 2000). This is in contrast to several *in vitro* studies that found significant effects of various antimicrobials on bovine neutrophil function, although the concentrations of antimicrobial applied to the neutrophils were supra-therapeutic and therefore difficult to extrapolate to the clinical setting (Ziv *et al.*, 1983; Nickerson *et al.*, 1985; Paape & Miller, 1990; Paape *et al.*, 1991; Hoebe *et al.*, 1997a,b, 1998).

As for diseased animals, calves with induced pneumonia treated with tilmicosin prior to bacterial inoculation had an increased incidence of apoptosis in neutrophils isolated from the lungs (Chin *et al.*, 1998). The significance of apoptosis of neutrophils in bovine respiratory disease has not been well established. Much of the damage that occurs to the lungs in *Pasteurella haemolytica* pneumonia in cattle can be attributed to the presence of neutrophils and their associated oxygen radicals and enzymes (Slocumbe *et al.*, 1985; Breider *et al.*, 1986, 1988). However, dead or apoptotic neutrophils cannot perform their microbicidal functions. The challenge in understanding the *in vivo* influence of antimicrobials on neutrophil function in *P. haemolytica* pneumonia is the potential for the bacteria themselves to affect neutrophil function, and the

consequent difficulty in separating these effects from those of the drug.

The purpose of this study was to investigate the effects of danofloxacin and tilmicosin on the function of circulating neutrophils and the extent of neutrophil apoptosis in the presence of induced *P. haemolytica* pneumonia. Circulating neutrophils are the source for recruitment of neutrophils to the lung, and assay of their function should be indicative of the functional ability of migrating neutrophils as they enter the lung, avoiding the potential local, but not systemic, effects of *P. haemolytica*. The other objective of this study was to evaluate the abilities of the two antimicrobials to ameliorate the extent of lung consolidation caused by *P. haemolytica*.

MATERIALS AND METHODS

The experimental protocol was approved by the Iowa State University Committee on Animal Care.

Animals

Angus-cross heifers, approximately 6 months of age weighing an average of 181.3 kg (SD = 19.0) on arrival, were purchased as a group from a single herd in Nebraska and shipped to allow 14 days of acclimation prior to the start of the study. Calves had been vaccinated with modified live viral respiratory vaccines; no *Pasteurella* vaccine was administered. There was no history of treatment with fluoroquinolone or macrolide antimicrobials prior to the start of the study. Animals were housed outdoors in concrete-floored pens with open front sheds as shelter, and were fed free choice hay and a grain supplement containing monensin sodium at a rate of approximately 150 mg/head/day.

Bacterial challenge

A frozen (−70 °C) stock culture of *P. haemolytica* A1 strain L101 isolated from a calf with pneumonia and then stored at −70 °C was used to prepare the inoculum as previously described, except that the final incubation was for 3 h (Lehmkuhl *et al.*, 1989). The broth culture was then adjusted to an approximate concentration of 1×10^8 cfu/mL. Concentrations of bacteria in the inoculum were verified after the inoculation procedure with standard plate counts.

An equine bronchoalveolar lavage catheter (Bivona, Inc., Gary, IN, USA) was passed through the nares into the trachea as far as possible into a caudal lung lobe, then withdrawn approximately 20 cm, and culture fluid (20 cc) was placed in the catheter, followed by approximately 180 mL of air.

Selection criteria

Animals were randomly selected from a group of 48 for *P. haemolytica* challenge, 18 during week 1 and 15 during week 2. At 20 h after bacterial challenge, 12 animals displaying a rectal

temperature of ≥ 40 °C and a clinical score of 1 or greater were randomly selected each week for treatment, resulting in a total of 24 challenged–treated animals (Animals not selected were treated appropriately with an antimicrobial). The clinical scoring system has been previously described (Perino & Apley, 1998), with a score of 0 – a normal animal, 1 – noticeable depression without apparent signs of weakness, 2 – marked depression with moderate signs of weakness without significantly altered gait, 3 – severe depression with signs of weakness such as significantly altered gait, and 4 – moribund and unable to rise.

Each week, the 12 selected animals were sorted by temperature and grouped into blocks of three. A random number from a uniform distribution on the interval (0, 1) was assigned to each animal, and the animals were sorted within the four temperature blocks by the random number. The first animal within each block was assigned to saline, the second to danofloxacin, and the third to tilmicosin.

Four animals were also selected each week at random to be nonchallenged, nontreated controls (NCH), for a total of eight NCH calves.

Clinical scoring

Clinical scoring was performed as described under selection criteria at 0, 24, 48 and 72 h after treatment by an investigator masked to treatment group identity.

Antimicrobials

Animals received one of the following treatments at 20 h after bacterial challenge: danofloxacin mesylate (Advocin 180; Pfizer, Inc., New York, NY, USA) (180 mg/mL) at a dose of 6 mg/kg subcutaneously (s.c.) in the left lateral neck, tilmicosin (Micotil; Elanco Animal Health, Indianapolis, IN, USA) (300 mg/mL) at a dose of 10 mg/kg s.c. in the left lateral neck, or saline (0.9%) in a volume equal to a dose of danofloxacin s.c. in the left lateral neck.

Blood sampling

Blood samples were collected at 3, 24 and 48 h after treatment for hematology and neutrophil isolation.

Hematology

Blood samples were collected via jugular venipuncture and placed in tubes containing EDTA (Vacutainer; Becton Dickinson and Co., Franklin Lakes, NJ, USA). An automated cell counter (Cell-Dyne 3500; Abbott Labs, Abbott Park, IL, USA) was used to measure total and differential leukocyte count, erythrocyte count, hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count, and mean platelet volume.

Neutrophil isolation

Blood samples were collected via jugular venipuncture, and neutrophils were isolated as previously described (Fajt *et al.*, 2000). Samples were suspended with Hanks balanced salt solution to a concentration of 5×10^7 cells/mL for use in the neutrophil assays.

Neutrophil assays

These assays have been previously described (Roth & Kaeberle, 1981a, 1994; Lukacs *et al.*, 1985; Fajt *et al.*, 2000). The assays performed included random migration under agarose, cytochrome C reduction, iodination, *Staphylococcus aureus* ingestion, chemotaxis, and antibody-dependent and antibody-independent cell-mediated cytotoxicity assay using radiolabeled (^{51}Cr) chicken erythrocytes.

Apoptosis was determined using a commercially available cell death detection kit (Cell Death Detection ELISA; Roche Molecular Biochemicals, Indianapolis, IN, USA). This kit is a sandwich ELISA, using anti-histone antibody, test sample containing nucleosomes, and anti-DNA peroxidase to measure photometrically the amount of histone-associated DNA fragments generated during apoptosis. Unstimulated levels of apoptosis were determined as was the percentage apoptosis after addition of camptothecin (0.1 and 1.0 $\mu\text{g/mL}$), an apoptosis-inducing agent.

Lung lesions

Animals were killed with an overdose of barbiturate. At necropsy, the lungs were removed from calves, and the percentage of consolidation of each lobe was visually estimated. Estimates were to the nearest 10%, except that lobes with <10% but >0% consolidation were estimated as 5%. Previously unpublished data (personal communication, Terry Skogerboe, Pfizer Animal Health, 1998) derived from bovine lungs obtained from a slaughterhouse were used to construct the following formula to calculate total lung consolidation:

Total percentage lung consolidation = $(0.053 \times \text{cranial segment of left cranial lobe \%}) + (0.049 \times \text{caudal segment of left cranial lobe \%}) + (0.319 \times \text{left caudal lobe \%}) + (0.043 \times \text{accessory lobe \%}) + (0.352 \times \text{right caudal lobe \%}) + (0.061 \times \text{right middle lobe \%}) + (0.060 \times \text{caudal segment of right cranial lobe \%}) + (0.063 \times \text{cranial segment of right cranial lobe \%})$.

Areas of consolidation were sampled for histopathology and bacterial culture.

Minimum inhibitory concentration determinations

Minimum inhibitory concentrations (MIC) of several antimicrobials were determined for *P. haemolytica* used for the bacterial challenge as well as for *P. haemolytica* isolated from the lungs of calves collected at necropsy. An automated broth-dilution method was used to measure MICs (Sensititre[®]; Trek Diagnostic Systems, Westlake, OH, USA). Custom plates containing several dilutions of the antimicrobials listed in Table 5 were used.

Statistical analysis

Unless otherwise stated, values reported are least-squares means and standard errors (SEM). The following variables were natural log transformed prior to analysis: neutrophil chemotactic index, MCV, lymphocyte absolute count, monocyte absolute count, RDW, mean platelet volume, and mononuclear white blood cell count. The least-squares means were then back-transformed after analysis. The repeatedly measured variables from the challenged animals (three treatment groups) including the neutrophil assay variables, apoptosis assay, and hematology results, were analyzed using a general linear repeated-measures mixed model (SAS Open VMS version 6.12 for an alpha, 1998; SAS Institute, Inc., Cary, NC, USA). If a significant difference was detected ($P \leq 0.05$) due to day of study or treatment effect, pairwise comparisons among treatments were made using *t*-tests. Animals in the three challenged groups were blocked by rectal temperature before treatments were assigned. Because NCH calves could not be included in the blocking scheme with the three challenged groups, they were compared only with the saline-treated calves via *t*-test, which used the SE from the statistical analysis of the challenged-treated groups. Statistical significance was defined as $P \leq 0.05$.

Calculations were performed for the neutrophil assays to determine the power to determine a statistical difference at the 0.05 level (Stroup, 1999). Power was calculated for each contrast performed within each neutrophil assay when no significant differences were found. In addition, the least significant difference required to detect a difference with 80% certainty at the 0.05 level was calculated for each contrast.

Percentage lung consolidation data was transformed using the arcsine square root transformation before analysis, and was then analyzed using a general linear mixed model. Pairwise comparisons were made among treatments using the *t*-test if a significant ($P \leq 0.05$) treatment effect was found. After analysis, the least-squares means were back-transformed for presentation.

RESULTS

Standard plate counts of the *P. haemolytica* challenge strain revealed a concentration of 2.9×10^8 cfu/mL in the first group and 1.7×10^8 cfu/mL in the second group of animals.

Of the 33 animals challenged with *P. haemolytica*, 28 exhibited the selection criteria, and all selected calves had clinical scores of 1 or 2. By 72 h after treatment, just prior to killing, two calves in the tilmicosin-treated group exhibited a clinical score of 0, with all others remaining at 1 or 2, although there were no significant differences among the treatment groups in body temperature (data not shown).

Neutrophil assays

For all of the neutrophil function assays (random migration, cytochrome C, iodination, *S. aureus* ingestion, chemotaxis, and cytotoxicity), there was a significant day-of-collection effect.

Table 1. The distance neutrophils migrated under agarose in a square milliliter (neutrophil random migration assay). Neutrophils were collected at 3, 24 and 48 h after treatment with danofloxacin, tilmicosin or saline (approximately 23, 44 and 68 h after challenge with *Pasteurella haemolytica*). NCH (nonchallenged group) is arithmetic mean; all other groups are least-squares means. Statistical analysis was performed to compare the three challenged–treated groups, and to compare NCH calves with saline-treated calves. The lower portion of the table shows the least significant difference (LSD) required between the challenged groups to show a statistical difference, along with the statistical power to detect a difference

Time after treatment	3 h	24 h	48 h
Saline	141.8 ± 15.1	47.5 ± 8.9	50.5 ± 8.7*
Danofloxacin	127.8 ± 15.1	38.9 ± 8.9	37 ± 8.7
Tilmicosin	106.5 ± 15.1	55.4 ± 8.9	46.3 ± 8.7
NCH	158.7 [†]	70.1 [†]	90.4 ^{*†}
Contrast	LSD [‡] (Power [§])	LSD [‡] (Power [§])	LSD [‡] (Power [§])
Saline vs. danofloxacin	54.2 (0.11)	20.1 (0.23)	18.6 (0.54)
Saline vs. tilmicosin	54.2 (0.46)	20.1 (0.20)	18.6 (0.10)
Danofloxacin vs. tilmicosin	54.2 (0.20)	20.1 (0.65)	18.6 (0.30)

*Differences significant at $P \leq 0.05$.

[†]SEs or CIs are not reported for the NCH group, because the SE used in the statistical analysis was from the repeated measures analysis of challenged–treated groups.

[‡]The absolute value of the difference in the least-squares means that would be required to declare a statistically significant difference at the 0.05 level with 80% certainty.

[§]The likelihood of detecting a difference between the group mean at $P \leq 0.05$.

There were, however, no significant differences among the challenged–treated groups (saline, danofloxacin, or tilmicosin-treated) for any of the neutrophil function assays at 3, 24 or 48 h after treatment.

When the NCH calves were compared with the saline-treated calves, there were no statistically significant differences between the groups for the neutrophil chemotaxis assay, the iodination assay, the unstimulated or stimulated cytochrome C reduction assay, or the antibody-dependent cell-mediated cytotoxicity assay.

Significant differences were found between the saline-treated calves and the NCH calves for the following assays: neutrophil random migration was significantly lower in the saline-treated calves than in the NCH calves at 48 h after treatment (Table 1). Neutrophils from the saline-treated calves displayed significantly higher *S. aureus* ingestion percentage than neutrophils from NCH calves at 24 h after treatment (Table 2). The percentage antibody-independent cytotoxicity was significantly higher for the saline-treated calves than for the NCH calves at 3 h after treatment (Table 3). There were no other significant differences between the saline-treated and the NCH calves.

There were no significant differences among the challenged–treated groups or between the NCH and the saline-treated groups for the cell death detection ELISA, whether apoptosis was stimulated or not.

Table 2. Percent radiolabeled *Staphylococcus aureus* ingested by neutrophils (*S. aureus* ingestion assay). Neutrophils were collected at 3, 24 and 48 h after treatment with danofloxacin, tilmicosin or saline (approximately 23, 44 and 68 h after challenge with *Pasteurella haemolytica*). NCH is arithmetic mean; all other groups are least-squares means with SEs. Statistical analysis was performed to compare the three challenged–treated groups, and to compare NCH calves with saline-treated calves. The lower portion of the table shows the least significant difference required between the challenged groups to show a statistical difference, along with the statistical power to detect a difference

Time after treatment	3 h	24 h	48 h
Saline	34.4 ± 2.3	29.6 ± 2.4*	18 ± 4.2
Danofloxacin	35.3 ± 2.3	30.9 ± 2.4	14 ± 4.2
Tilmicosin	34 ± 2.3	30.1 ± 2.4	18.4 ± 4.2
NCH	29.9 [†]	20.3 ^{*†}	7.1 [†]
Contrast	LSD [‡] (Power [§])	LSD [‡] (Power [§])	LSD [‡] (Power [§])
Saline vs. danofloxacin	9.56 (0.06)	10.0 (0.07)	17.2 (0.10)
Saline vs. tilmicosin	9.56 (0.05)	10.0 (0.05)	17.2 (0.05)
Danofloxacin vs. tilmicosin	9.56 (0.07)	10.0 (0.06)	17.2 (0.11)

See footnote of Table 1 for explanation of symbols.

Hematology

There were no significant differences for total white blood cell count or absolute neutrophil, lymphocyte, monocyte, eosinophil or basophil counts among the challenged groups including saline-treated calves. Total white count was significantly higher for the saline-treated calves compared with the NCH calves at 24 and 48 h after treatment, for absolute neutrophil count at 3, 24, and 48 h after treatment, and for absolute monocyte count at 24 and 48 h after treatment (see Table 4).

There were no significant differences among the challenged–treated groups or between the NCH and the saline-treated groups for total erythrocyte count, hemoglobin, MCV, MCH, or RDW. The mean hematocrit for danofloxacin-treated calves ($31.5 \pm 1.0\%$) was significantly lower than saline- ($35.5 \pm 1.0\%$, $P = 0.006$) or tilmicosin-treated ($35.9 \pm 1.0\%$, $P = 0.002$) calves at 24 h after treatment, and than that for tilmicosin-treated calves at 48 h after treatment ($29.7 \pm 1.0\%$ vs. $32.5 \pm 1.0\%$, $P = 0.05$). The difference between saline- ($32.5 \pm 1.0\%$) and danofloxacin-treated ($29.7 \pm 1.0\%$) calves also approached significance at 48 h ($P = 0.0522$), with danofloxacin-treated calves being lower.

There were no significant differences among challenged–treated groups or between NCH and saline-treated groups for mean platelet volume. Total number of platelets did not differ significantly among challenged–treated groups, but was significantly lower at 3, 24 and 48 hours after treatment in the saline-treated group compared with the NCH group (data not shown).

Lung consolidation and bacteriology

No significant differences for percentage of lung consolidation were found among treatment groups or among blocks of animals

Table 3. Percentage lysis of radiolabeled chicken erythrocytes (cRBC) without anti-cRBC antibody. Neutrophils were collected at 3, 24 and 48 h after treatment with danofloxacin, tilmicosin or saline (approximately 23, 44 and 68 h after challenge with *Pasteurella haemolytica*). NCH is arithmetic mean; all other groups are least-squares means with SEs. Statistical analysis was performed to compare the three challenged–treated groups, and to compare NCH calves with saline-treated calves. The lower portion of the table shows the least significant difference required between the challenged groups to show a statistical difference, along with the statistical power to detect a difference

Time after treatment	3 h	24 h	48 h
Saline	20.5 ± 6.1*	10.2 ± 4.2	10.3 ± 3.9
Danofloxacin	21.6 ± 6.1	9.8 ± 4.2	13.1 ± 3.9
Tilmicosin	22.8 ± 6.1	11 ± 4.2	10.8 ± 3.9
NCH	2.3* [†]	1.4 [†]	0.7 [†]
Contrast	LSD [‡] (Power [§])	LSD [‡] (Power [§])	LSD [‡] (Power [§])
Saline vs. danofloxacin	22.6 (0.05)	13.4 (0.05)	12.0 (0.10)
Saline vs. tilmicosin	22.6 (0.06)	13.4 (0.05)	12.0 (0.05)
Danofloxacin vs. tilmicosin	22.6 (0.05)	13.4 (0.06)	12.0 (0.08)

See footnote of Table 1 for explanation of symbols.

Table 4. Total leukocyte counts showing statistically significant differences between saline-treated and NCH calves. Peripheral blood samples were collected at 3, 24 and 48 h after treatment with saline (approximately 23, 44 and 68 h after challenge with *Pasteurella haemolytica*). Total white count and neutrophil count mean are least-squares means for saline-treated calves with SEs, and arithmetic mean for NCH calves. Monocyte count mean are geometric mean and 95% CIs. Statistical analysis was performed to compare the three challenged–treated groups, and to compare NCH calves with saline-treated calves

Time after treatment	3 h	24 h	48 h
Total white count (thousands)			
Saline	12.8 ± 1.7	12.4 ± 1.3*	10.9 ± 0.9*
NCH	8.4 [†]	6.8* [†]	7.6* [†]
Neutrophil count (thousands)			
Saline	8.0 ± 1.2*	7.6 ± 1.0*	5.5 ± 0.7*
NCH	3.7* [†]	2.4* [†]	2.6* [†]
Monocyte count (thousands)			
Saline	1.2 (1.0–1.6)	1.7 (1.3–2.2)*	1.7 (1.3–2.2)*
NCH	0.9 [†]	0.9* [†]	1.1* [†]

*Differences between saline-treated and NCH calves significant at $P \leq 0.05$.

[†]Standard errors or CIs are not reported for the NCH group, because the SE used in the statistical analysis was from the repeated measures analysis of challenged–treated groups.

(blocked by temperature at the time of treatment). Total percentage of lung consolidation in individual animals ranged from 2.02 to 29.6%, with mean of 18.1, 14.7, and 13.0% for saline, danofloxacin, and tilmicosin-treated animals, respectively.

Pasteurella haemolytica was isolated from seven of the eight saline-treated, eight of the eight danofloxacin-treated, and five of the eight tilmicosin-treated calves. Growth from one tilmicosin-

Table 5. Minimum inhibitory concentrations (MICs) in micrograms per milliliter of the challenge inoculum of *Pasteurella haemolytica* to antimicrobials, as evaluated using the Sensititre[®] susceptibility testing system

Antimicrobial	MIC (µg/mL)
Amoxicillin/clavulanic acid	≤ 1.0/0.5
Cefquinome	≤ 0.06
Ceftiofur	≤ 0.06
Colistin	≤ 0.25
Danofloxacin	0.06
Enrofloxacin	0.03
Gentamicin	≤ 2.0
Oxytetracycline	>64
Spectinomycin	32
Spiramycin	128
Tilmicosin	4
Trimethoprim/sulfadiazine	0.5/9.5
Tylosin	64

treated calf revealed no *P. haemolytica*, but low numbers of colonies of *S. epidermidis* and *P. multocida*. The MICs for the challenge isolate are shown in Table 5. The MICs for all of the lung isolates were the same as the challenge inoculum with two exceptions: the MIC for spectinomycin was 16 µg/mL for three isolates, and the MIC for oxytetracycline was 64 µg/mL for two isolates, 1.0 µg/mL for one isolate, and ≤0.5 µg/mL for three isolates.

Histopathologic examination of samples from all lungs revealed changes consistent with subacute pneumonic pasteurellosis such as foci of necrosis surrounded by necrotic neutrophils, fibrinous bronchopneumonia, suppurative bronchopneumonia, and atelectasis.

DISCUSSION

The number of animals displaying the desirable clinical characteristics after challenge with *P. haemolytica* (28 of 33 or 85%) suggests that this induction model will continue to be useful in pharmaceutical and other trials. As might be expected in the clinical setting, some animals began to display clinical scores of 0 by 72 h after treatment with an antimicrobial.

The lack of significant differences in the neutrophil function assays in the challenged–treated groups is consistent with the authors' previous findings in healthy beef calves, where no differences were noted for the same neutrophil function assays among saline-, danofloxacin-, or tilmicosin-treated calves (Fajt *et al.*, 2000). As danofloxacin and tilmicosin administered to healthy animals did not affect neutrophil function, it was hypothesized that the addition of induced *Pasteurella* pneumonia to the scheme should not affect neutrophil function differentially between the two antimicrobials. These results are in contrast to other researchers (Chin *et al.*, 1998), who found that neutrophils isolated from the lungs of tilmicosin-treated *P. haemolytica*-challenged calves exhibited increased apoptosis at 3 h after treatment compared with sham-treated animals (although there were no differences at 24 h after treatment). One possibility is

the source of neutrophils: the Chin study used neutrophils isolated from the lungs whereas this study examined circulating neutrophils. It is also possible that the preinduction treatment with tilmicosin in their study reduced the growth and metabolism of *P. haemolytica* at 3 h after challenge, resulting in less *P. haemolytica*-induced apoptosis, rather than a direct effect of tilmicosin on neutrophil apoptosis.

It should be noted here that because concurrent pharmacokinetic studies would have been cost-prohibitive for the number of animals in this trial, drug serum concentration data were not gathered. However, because the doses used were as labeled on the approved products, an assumption of 'therapeutic concentrations' was made; one of the purposes of this study was to assess effects of the antimicrobials at labeled doses rather than at unattainable or potentially toxic suprathreshold doses. It is certainly possible, although unlikely, that the lack of significant differences in the neutrophil function assays was due to subtherapeutic concentrations of antimicrobials. Given that the mean time-at-peak-concentration (t_{max}) was 0.5 h for cattle given the same dose of tilmicosin (but intramuscular) as in this study (Modric *et al.*, 1998) and 1 h for cattle given a slightly lower dose (5 mg/kg rather than 6 mg/kg, also intramuscular) of danofloxacin (Mann & Frame, 1992), and given that the mean elimination half-life in those same studies was 30 h for tilmicosin (Modric *et al.*, 1998) and 2.9 h for danofloxacin (Mann & Frame, 1992), neutrophil collection at 3 h represents a reasonable time to assess relatively high concentrations of the antimicrobials, i.e., high within the range of concentrations manifested at therapeutic dosages.

Because no significant differences were found among the challenged-treated groups for the neutrophil assays, the power to detect these differences was calculated. Using a reasonable number of animals (eight per treatment group), the power ranged from 0.05–0.65 depending on the assay. There are many examples in the literature in which these assays had the power to detect differences using eight to 10 animals (Coe *et al.*, 1992; Flaming *et al.*, 1993, 1997). Because power is often not reported in studies similar to this one, there is no basis for comparison. However, taking into account the expensive nature of the animals and the neutrophil assays as well as the laboratory logistics, adding more subjects to the experimental design would have been cost-prohibitive.

The only differences in the neutrophil function assays noted among the groups were between the saline-treated and the NCH calves. The fact that the only differences were between the challenged-saline-treated calves and the NCH calves suggests that these differences are caused by the *P. haemolytica* challenge, not antimicrobial effects, perhaps as a result of circulating factors associated with the infection as the neutrophils were collected from the peripheral circulation. Challenged calves had higher core body temperatures as measured via radiotelemetry (data not shown), suggesting pro-inflammatory cytokine release, which could effect changes in neutrophil function (Roth, 1994).

As expected, there were no significant differences among the challenged-treated groups for the majority of the hematology parameters, but there were significant differences between the saline-treated and the NCH calves. The NCH calves were not

challenged with *P. haemolytica* and would therefore not be expected to experience the increased leukocyte and neutrophil count typically associated with this infection (Vestweber *et al.*, 1990). One exception to the lack of significant differences in hematology was the lower hematocrit associated with the danofloxacin-treated group, although the hematocrit remained within normal limits for the laboratory (24–46%). This phenomenon was also found in the authors' previous report on danofloxacin in healthy calves (Fajt *et al.*, 2000), although it has not been reported elsewhere for danofloxacin.

The other exception to the lack of significant differences among groups in hematology was the finding of lower platelet counts in saline-treated animals than in the NCH calves. This may be the result of increased disappearance of platelets associated with the pathophysiology of *P. haemolytica* infection, which results in damage to endothelial cells, hemorrhage and therefore platelet activation and subsequent microthrombosis (Weekly *et al.*, 1998). Other investigators found decreased platelet survival at 6 h postinoculation with *P. haemolytica* (Rashid *et al.*, 1997).

The lack of significant differences among the challenged-treated groups in percentage of lung consolidation can be partially explained by the individual variability of consolidation. Using this same induction model, the authors found a range of 5.0–37.5% consolidation in nontreated animals (unpublished data). Additionally, the time of necropsy (72 h after treatment) may not have allowed sufficient time for lesion healing that might have occurred as a result of antimicrobial therapy, or sufficient time for lesions to develop. It is also possible that the moderate infection produced by this induction model did not allow significant differences to manifest, whereas a more severe challenge model might have.

The changes in MICs from challenge inoculum to lung isolate may be due to passage in the lungs. As no fingerprinting or strain analysis was performed on the isolates, this could not be confirmed. The MICs were similar enough across the majority of antimicrobials; so the challenge strain was likely the same as the isolated strain.

Overall, these results suggest that there are no major, clinically significant effects of either danofloxacin or tilmicosin on circulating neutrophil function and apoptosis in induced *P. haemolytica* pneumonia. However, there is a significant effect of *P. haemolytica* infection that results in changes in circulating neutrophil function in the absence of antimicrobial administration.

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