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Virginia R. Fajt
Iowa State University

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The effects of danofloxacin and tilmicosin on peripheral neutrophils in healthy cattle, on peripheral neutrophils in cattle with induced *Pasteurella haemolytica* pneumonia, and on body temperature measured via radiotelemetry in cattle with induced *Pasteurella haemolytica* pneumonia

by

Virginia R. Fajt

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ABSTRACT

Three studies are described in this report. In the first study, the effects of danofloxacin and tilmicosin on neutrophil function were examined in healthy mixed-breed weaned beef heifers. Neutrophils were isolated from peripheral blood samples pre- and post-treatment. The neutrophil function assays performed were: random migration under agarose, Cytochrome C reduction, iodination, *Staphylococcus aureus* ingestion, chemotaxis under agarose, and antibody-independent and antibody-dependent cell-mediated cytotoxicity. The results suggest that at therapeutic drug concentrations, danofloxacin and tilmicosin have little clinical effect on bovine neutrophil function. In the second study, a model was developed for the induction of pneumonic pasteurellosis (using 6 x 10^9 CFU of a log-phase culture of *Pasteurella haemolytica* intrabronchially) in weaned, pre-conditioned beef calves that consistently produced rectal temperatures of at least 40°C 24 hours after bacterial challenge, a clinical score of ≥1, and measurable lung consolidation. In the third study, *Pasteurella* pneumonia was induced in weaned beef heifer calves using the model from the second study, and calves were treated with danofloxacin or tilmicosin. Neutrophils were collected at 3, 24 and 48 hours after treatment. Neutrophil function assays were as performed in study 1, and apoptosis was determined using a cell death detection kit. The results suggest that danofloxacin and tilmicosin have no clinically significant effects on neutrophil function or apoptosis. There were also no significant differences in percent lung consolidation among treatments. Significant differences were found between non-challenged calves and the challenged non-treated calves in several neutrophil assays, which were attributed to an effect of the *Pasteurella* infection. Body temperature was also examined in this study via intravaginally implanted radiotransmitters. Temperatures were monitored prior to challenge

until necropsy at 72 hours after treatment. The areas under the curve of the temperature-time plot (and over a baseline temperature established for each animal) calculated for 3-hour intervals were not significantly different for any of the time intervals when challenged animals were compared. Analysis of the mean 3-hour interval temperatures showed significantly higher temperatures for saline-treated as compared to antimicrobial-treated animals for the majority of the time intervals, but no differences were found between the danofloxacin- or tilmicosin-treated animals.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is presented in an introductory chapter, four chapters corresponding to papers prepared for submission for publication, and a final chapter of general conclusions. The chapters are presented in the order in which the experiments were performed, but also in an order that is logical in terms of how to interpret the data gathered. The second chapter presents the study that evaluated the effects of the two antimicrobials on the functions of circulating peripheral neutrophils in healthy cattle. The third chapter is a discussion of the *Pasteurella* pneumonia induction model and its development. This study was needed to verify that pneumonia could be reliably and consistently induced in the age and class of cattle utilized. The fourth chapter presents the study in which the effects of the two antimicrobials were evaluated in animals with pneumonia. The interpretation of these results was facilitated by the completion of the first study, since healthy and diseased animals could then be compared. The fifth chapter presents the body temperature information collected via radiotelemetry at the same time neutrophil function information was gathered in the third chapter. This information is a useful addition to the literature on *Pasteurella* pneumonia in characterizing the temperature response of cattle, and is also presented as another method of comparing therapies used in the treatment of pneumonia caused by *Pasteurella haemolytica* in cattle.

The co-authors on the submitted manuscripts contributed in the following ways: Michael D. Apley was the major professor and involved in all aspects of the studies including study design, data collection and analysis. James A. Roth was the immunologist involved in study design for all studies involving neutrophil function, and personnel in his laboratory

performed all the neutrophil assays. Dagmar E. Frank performed the majority of the neutrophil assays including the various attempts at isolating neutrophils from lungs in Chapter 2. Kim A. Brogden prepared and administered the *Pasteurella* cultures for Chapters 2-4. Terry L. Skogerboe was the Study Monitor from Pfizer Animal Health, the sponsoring organization, and was involved in study design, data collection, and data analysis. Valerie K. Karle and Arthur D. Dayton were the statisticians from Pfizer Animal Health and were involved in study design and data analysis.

Literature Review

As long as antimicrobials have been available clinically, there has been interest in their effects on the immune system. The major classes of effects include their toxicity to neutrophils, their ability to modify bacterial virulence at subtherapeutic or supratherapeutic concentrations, and their modulation of phagocyte function.[1] The focus of the studies reported in this dissertation was the third type of effect, the modulation of phagocyte function, specifically the neutrophil.

Neutrophils are one arm of the non-specific immune system and are recruited to the site of bacterial and viral infections in advance of the humoral and cell-mediated responses.[2] In addition, once humoral or cell-mediated immunity is stimulated, neutrophil activity may be enhanced.[2] Neutrophils function in several ways to fight infectious agents: They kill microbes via oxygen-dependent and oxygen-independent mechanisms, and they kill cells infected with agents such as viruses via antibody-dependent and antibody-independent mechanisms.[2] The oxygen-independent killing mechanisms are mostly related to the cytoplasmic granules found in neutrophils, some examples being proteases, elastases and glutathione peroxidase. The oxygen-dependent killing mechanism utilizes a membrane-

associated enzyme complex to convert oxygen to superoxide anion which is subsequently converted to hydrogen peroxide, hydroxyl radical, and singlet oxygen, all of which are bactericidal. Hydrogen peroxide is also utilized via the myeloperoxidase-hydrogen peroxide-halide system in the halogenation and oxidation of bacterial surface components resulting in potent bactericidal activity.[2]

Many methods of measuring the functional abilities of neutrophils have been studied. These methods all require the isolation of neutrophils from the blood or other tissues. Characterizing the effects of various compounds or cytokines on neutrophils is accomplished either by isolation of the neutrophils and subsequent application of the treatment (*in vitro*) or by application of the treatment in the animal or human and subsequent isolation of the neutrophils (*ex vivo*), since functional ability cannot be assessed to a great extent in vivo. In general, the following functions of neutrophils can be evaluated through laboratory efforts: the ability to migrate to the site of infection (chemotaxis), the ability to phagocytose bacteria, the ability to kill infectious agents directly through oxygen-dependent and oxygen-independent mechanisms, and the ability to kill cells infected with infectious agents. The ability to migrate can be evaluated by measuring migration toward a chemotactic substance under agarose or using a Boyden chamber (upper and lower chambers separated by a membrane).[3] The ability to phagocytose can be evaluated using various pathogens, such as *Staphylococccus aureus*, with the amount phagocytosed measured by either staining or radiolabeling the pathogen.[3,4] Bacterial killing can be assessed by measuring actual bacteria killed. The oxygen-dependent bacterial killing mechanisms can be evaluated by measuring the production of superoxide anions indirectly via chemiluminescence, the reduction of cytochrome C or the reduction of nitroblue tetrazolium

reduction. The myeloperoxidase-hydrogen peroxide-halide system (oxygen-independent killing) can be evaluated by measuring iodination of opsonized zymosan or by directly measuring the activity of myeloperoxidase once it is released from the neutrophils.[3,4] The ability to kill cells (antibody-dependent and antibody-independent cell-mediated cytotoxicity) can be evaluated using radio-labeled chicken red blood cells as targets in the presence or absence of specific antibodies.[5]

Utilizing these assays, the effects of various antimicrobials on the functional ability of neutrophils have been evaluated. In humans, these efforts have been both *in vitro* and *ex vivo*, whereas in cattle, the majority of the studies have been *in vitro*. The effects of the major categories of antimicrobials on human neutrophils have been reviewed and will not be completely summarized here.[3,6] However, the effects of the two classes of antimicrobial (macrolides and fluoroquinolones) used in the studies presented in this dissertation will be summarized herein, both for human neutrophils and for cattle. The selection of antimicrobials for the studies reported in this dissertation was based on several factors: 1) the interest of the sponsoring agency in their product, danofloxacin, 2) the fact that some work suggested a modulating role of the macrolide tilmicosin on neutrophil function, and 3) the fact that both drugs are approved for the treatment of respiratory disease in cattle, tilmicosin in the US and Europe, and danofloxacin in South America and Europe.

The macrolide antimicrobials have been extensively studied for their ability to alter neutrophil function, particularly since they possess an ability to enter phagocytes easily. Although differing techniques used to measure function may alter the results, in general, the oxidative functions of human neutrophils have been shown to be inhibited *in vitro* by roxithromycin, dirithromycin, and erythromycin.[3] Erythromycin, a 14-membered ring

macrolide, inhibits oxidant production[6], but 16-membered ring macrolides have been shown not to inhibit oxidative functions and may in fact produce an increased response.[3] Other functions that have been shown to be enhanced by macrolides are phagocytosis and bacterial killing. Increased chemotaxis has been noted with some macrolides when measured using a Boyden chamber, but no effects were noted when measured under agarose.[3] These results concur with the *ex vivo* results with therapeutic doses of erythromycin which increased chemotaxis.[3]

Studies on macrolides and cattle neutrophils *in vitro* have shown no effect on phagocytosis with erythromycin, tylosin, or spiramycin. [7,8] The oxidative activity (measured via chemiluminescence) of cattle neutrophils was significantly reduced by erythromycin and spiramycin at 1000 μg/ml but not at lower (physiologic) concentrations.[9] Production of superoxide anion (measured via cytochrome C reduction) was not reduced by erythromycin or spiramycin.[9]

In humans, some work shows that fluoroquinolones do not appear to inhibit the respiratory burst.[3,10,11] However, subsequent studies have shown both an enhancement of oxidative activity (measured via chemiluminescence or superoxide anion production)[12-15], and a reduction in superoxide anion production.[16] Most work indicates that in humans fluoroquinolones do not affect chemotaxis, with the drugs tested being nadifloxacin, norfloxacin, ofloxacin, pefloxacin, cinoxacin, enoxacin, and ciprofloxacin.[16-18] In contrast, fleroxacin was shown to significantly reduce chemotaxis.[19] Differing results have been reported as to the effects of fluoroquinolones on phagocytosis: Enoxacin and lomefloxacin were shown to enhance phagocytosis of *Staphylococcus aureus*; ciprofloxacin, pefloxacin, norfloxacin, and ofloxacin were shown to suppress phagocytosis of *Candida albicans*; and

enoxacin and ciprofloxacin were shown to have no effect on phagocytosis of *S. aureus*. There has also been a suggestion of a synergistic effect between oxygen-dependent mechanisms and the fluoroquinolones in the elimination of the intracellular pathogen *S. aureus*, although non-treated neutrophils were not examined in this paper.[20] Apparently, many of the effects of fluoroquinolones are drug dependent rather than antimicrobial-class dependent.

In cattle neutrophils, several fluoroquinolones, including norfloxacin, pefloxacin, and ciprofloxacin, have shown no effect on phagocytosis.[8] The oxidative activity (measured via chemiluminescence) was significantly reduced by danofloxacin (at 100 and 1000 μg/ml), but was significantly increased by enrofloxacin (at 10 and 100 μg/ml).[9] Superoxide anion production (measured via cytochrome C reduction) was significantly reduced by danofloxacin at 1000 μg/ml.[9] Danofloxacin significantly reduced myeloperoxidase activity at 1000 μg/ml.[21]

The majority of the studies described above on the effects of antimicrobials on neutrophils were conducted in the absence of on-going infectious processes; neutrophils were generally collected from healthy animals. This approach allows the delineation of the effects of the antimicrobials themselves, but fails to address the potential effects on neutrophils in diseased animals. The problem is that infectious agents on their own can have considerable effects on neutrophils in the absence of an antimicrobial. The problem is confounded even further by the fact that, obviously, infectious processes have potent effects on the immune system, which can have subsequent effects on neutrophil function. This complication aside, we must at least examine the overall effects of infectious agents and the immune response on neutrophil function in the absence of treatment. In this dissertation, the infectious agent

examined was *Pasteurella haemolytica* as a cause of respiratory disease in cattle so the discussion will be limited to that agent.

To put in perspective the importance of respiratory disease in beef cattle, it has been reported as the most costly disease, with an estimated annual loss approaching $1 billion, and an estimated annual cost of prevention and treatment of $3 billion.[22] The losses occur due to actual death loss as well as loss of performance: average daily gain of animals with lung lesions caused by Bovine Respiratory Disease has been shown to be significantly lower than animals with no lung lesions.[23,24] Bovine Respiratory Disease (BRD) refers to a syndrome that has been associated with several viruses and bacteria in conjunction with some compromise to the respiratory defense system caused by environmental, nutritional and management factors. The most commonly isolated bacterial pathogens in feedlot cattle, and the pathogens for which treatment with an antimicrobial is instituted, are *Pasteurella haemolytica* and *Pasteurella multocida* with the latter more commonly isolated from younger cattle.[22]

Because of the importance of this disease complex in cattle production, hundreds of trials have been conducted to elucidate its pathogenesis, prevention, diagnosis, and treatment. Many of these trials are performed using experimental models of the disease rather than the naturally occurring disease in order to control elements such as the timing of treatment. Therefore, there are on-going efforts to develop disease models that more closely mimic the syndrome as it appears in the clinical setting. These induction models have included applications of a stressor prior to instillation of bacteria, e.g., mechanical (acetic acid,[25] cold water,[25] exercise[26]) and viral,[27] as well as numerous methods of instillation of bacteria

(aerosol,[28] transtracheal via needle[29] or catheter,[30] transthoracic via needle,[31] intrabronchial via endoscopy[32] or some type of endobronchial tube[33]).

For the purpose of our study, we needed a *Pasteurella* pneumonia induction model that fulfilled the following criteria: It must be applicable to weaned beef cattle approximately 6 months in age, it must be fast, consistent and reproducible, it must not require multiple pathogens or multiple applications of bacteria, it must not require knowledge of previous exposure (i.e., unknown *P. haemolytica* titres), it must not require anesthesia, and it must not require specialized equipment such as endoscopy. In addition, we had an interest in using a well-defined clinical case definition in order to contribute to a continuing effort to standardize clinical trials for pharmaceutical agents. One method of standardizing case definitions of bovine respiratory disease is to observe animals from a moderate distance and assign a clinical score based on an established scale[34] from 0-4: In this scoring system, 0 is normal with no clinical signs of disease, 1 is noticeable depression without signs of weakness, 2 is marked depression with moderate signs of weakness, 3 is severe depression with signs of weakness such as altered gait, and 4 is moribund and unable to rise. At the selected time of 24 hours after instillation of the inducing bacteria, an animal was considered to have respiratory disease if it had a clinical score of 1 or greater and a rectal temperature of 40°C or greater. Therefore, we performed a separate study (reported in Chapter 3) to develop and verify the model we intended to apply.

The pathogenesis of BRD is complicated even if we limit the discussion to *Pasteurella haemolytica* infection, and includes interactions of neutrophils, macrophages, lymphocytes, cytokines, adhesion molecules, and antimicrobial peptides. However, in all stages of the bacterial infection, the neutrophil plays a significant role, both in fighting

infection and in contributing to lung damage. There is evidence that neutrophil-depleted calves have decreased lung damage when experimentally infected with *P. haemolytica*, suggesting neutrophil enhancement of lesions.[35] Conversely, there is evidence that neutrophil-depleted calves have more widespread lesions than normal calves, implying that the role of the neutrophil in the pathogenesis of *P. haemolytica* infection is complicated.[36] In addition, it appears that not all of the extensive damage to the lungs can be blamed on neutrophils, since neutrophil-depleted animals still have significant disease.[37] However, it can be stated that neutrophils play a significant role in the pathogenesis of the *P. haemolytica* infection associated with BRD.

Because of this intimate association of neutrophils with *Pasteurella* infection, the effect of the bacteria directly on neutrophil function becomes important. And as mentioned above, if we are going to examine the effect of antimicrobials on neutrophil function in *Pasteurella* infection, we need to understand the effects of *Pasteurella* alone. It is known that the leukotoxin produced by *P. haemolytica* is cytotoxic to neutrophils, suggesting a virulence mechanism advantageous to the bacteria.[38] In addition, *P. haemolytica* and its products have been shown to have the following *in vitro* effects on the functional abilities of neutrophils: Culture fluid from *P. haemolytica* has been shown to induce chemotaxis,[39,40], as have isolated outer membrane proteins,[41] whereas *P. haemolytica* lysate or cultures had no effect on chemotaxis.[42] Oxidative activity (measured by chemiluminescence or cytochrome C reduction) has been shown to be induced by culture fluid from *P. haemolytica*[39], by washed *P. haemolytica* cultures,[43] and by isolated leukotoxin in most cases.[44-47] In some experiments, the activation of the oxidative response was followed by rapid diminution of the response, when isolated leukotoxin[46,48] or washed *P. haemolytica* cultures were used.[49]

Extracts of *P. haemolytica*, including lipopolysaccharide,[50] purified capsular polysaccharide,[51] and outer membrane proteins, have been shown to decrease phagocytosis.[41] Comparatively few data are available on the effects of *P. haemolytica* infection *in vivo* on neutrophils. Chemotaxis was found to be diminished in animals with induced *P. haemolytica* pneumonia, but no other differences were noted.[52]

In the context of neutrophils and their association with the destruction of lung tissue, it has been suggested that if neutrophils could be induced to undergo a controlled death such as apoptosis rather than a relatively uncontrolled death leading to release of toxic metabolites such as occurs with necrosis, the damage to the lungs might be reduced.[53,54] In addition, attenuation of apoptosis has been shown to reduce inflammation in a model of endotoxin-induced liver injury.[55] On the other hand, loss of neutrophils at a critical stage could lead to a diminished immune response and result in overwhelming infection as occurs in animals with defective or depleted neutrophils.[36] Perhaps apoptosis is beneficial only at a certain stage in the infectious process. Nonetheless, it is an area of immunology that has received considerable attention recently.

Apoptosis has been called programmed cell death and is a highly regulated process resulting in cell shrinkage or splitting into vesicles with little spillage of cell contents.[54] Typical features of apoptosis are membrane blebbing, nuclear condensation, and DNA fragmentation.[56] Bacterial pathogens have differing effects on apoptosis, with some species inducing and some inhibiting apoptosis in leukocytes.[56] *Pasteurella haemolytica* has been shown to induce apoptosis *in vitro*[57-59] and *in vivo* in an induced pneumonia model.[60]

Whether or not apoptosis is desirable in the context of *P. haemolytica* pneumonia may not be answered yet, but its mere presence in this disease suggests that we should

evaluate the effects of administered compounds such as antimicrobials on this aspect of the immune process. One study suggested that tilmicosin administered in experimental *P. haemolytica* pneumonia induced apoptosis of neutrophils.[30] These investigators suggest an anti-inflammatory benefit to tilmicosin, although this remains to be borne out. The difficulty with this type of *ex vivo* study is that the effects of the antimicrobial are confounded with the effects of the *Pasteurella*. Our answer to help resolve those effects was to perform two *ex vivo* studies using tilmicosin and another antimicrobial, danofloxacin: one in healthy animals (Chapter 2) and one in pneumonic animals (Chapter 4). This method could potentially shed light on whether the antimicrobials had direct effects on apoptosis and neutrophil function.

Because bacterial disease was induced in calves in this study, there was an opportunity to evaluate other clinical findings in the animals. As evidenced by the clinical criteria used in these experiments, it is well known that body temperature rises as a result of infection with *Pasteurella haemolytica*. Bacterial infections in general result in the release of pyrogenic cytokines such as IL-1, tumor necrosis factor and IL-6 from macrophages and other cells into the general circulation. It is believed that these cytokines act directly or indirectly on the vascular network close to the hypothalamus to produce prostaglandin E (PGE) via the arachidonic acid cascade.[61] PGE acts on the thermoregulatory center to cause a change in the set-point, resulting in systemic responses that increase body temperature such as vasoconstriction and shivering.[62] The measurement of body temperature is therefore used to monitor whether infection is occurring or whether the infection is being cleared.

In addition to the rectal temperatures measured at the time of treatment of animals, a remote method of evaluating body temperature via radiotelemetry was used in these studies. Radiotelemetry permits monitoring of body temperature without requiring the handling of

animals. In cattle, methods to monitor body temperature were developed in the 1960s[63,64] and subsequently refined over time. Now available to the scientist without an electronics and radio background are self-enclosed hermetically sealed transmitters that can be placed virtually anywhere in an animal, from the peritoneum to the vagina.[65] Systems are commercially available that allow automatic downloading of temperature data onto computer hard disk.[i] In addition, advancements in computer technology now allow the handling of large amounts of data using a desktop system, so that temperature can be recorded as often as desired and then be analyzed on the average personal computer.

Core body temperature has been measured in normal cattle to evaluate estrual patterns of temperature in dairy[66,67] and beef cows:[68] Body temperature rises at least 0.3°C for at least 3 hours during ovulation. Core body temperature response in beef and dairy cattle to climate such as heat in the summer[65,69,70] or cold in the winter[71] has been evaluated. It appears that in feedlot steers, there is a circadian pattern to core body temperature. In the winter, body temperature minima were at approximately 8 a.m. and maxima at approximately 7 p.m.[71] In the summer, the peak and trough temperatures occurred at similar times (8 a.m. and 6:30 p.m.), but daily minimum temperatures actually decreased slightly with an increase in ambient temperature, suggesting a compensatory mechanism to promote heat loss at night in anticipation of increased ambient temperatures during the day.[69] The importance of normal body temperature variation becomes evident when attempts are made to evaluate body temperature during disease.

The use of continuously recorded body temperature in response to disease in cattle has been more limited. The only reports found were of induced clinical mastitis in dairy

[i] Datacol5® Large Animal Monitoring Software, Minimitter Co., Sunriver, Oregon

cows.[72,73] The temperature response to *Pasteurella* pneumonia and its treatment has been evaluated via rectal temperature. However, convenience and handling facilities dictate limited data collection, particularly in the case of larger calves such as those used in our studies (average weight of approximately 400 lbs.). In addition, it could be argued that handling animals many times a day could affect their response to treatment due to additional stress. Therefore, our study on the effects of induced *Pasteurella* pneumonia in beef calves and its treatment on neutrophil function was expanded to include the evaluation of body temperature. It was hoped that continuously recorded body temperature might be a valuable tool in the assessment of response to pharmaceutical products with potential expansion to vaccine efficacy.

The major objectives of the studies reported in the following chapters were as follows:

(1) To characterize the effects on *ex vivo* neutrophil function of two antimicrobials, danofloxacin and tilmicosin, in healthy weaned beef heifers

(2) To develop a model of inducing *Pasteurella haemolytica* pneumonia in weaned beef heifers that resulted in an adequate number of animals exhibiting the desired case criteria

(3) To characterize the effects on *ex vivo* neutrophil function of danofloxacin and tilmicosin in weaned beef heifers with induced *P. haemolytica* pneumonia

(4) To characterize the effect of *P. haemolytica* pneumonia on continuously recorded body temperature of weaned beef heifers, and to determine if this method of monitoring can be used in comparative trials of pharmaceuticals or biologicals.

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CHAPTER 2. EFFECTS OF DANOFLOXACIN AND TILMICOSIN ON CIRCULATING NEUTROPHILS IN BEEF HEIFERS

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Virginia R. Fajt, Michael D. Apley, James A. Roth, Terry L. Skogerboe, Valerie K. Karle, Arthur D. Dayton

Abstract

The *in vivo* effects of two antimicrobials, danofloxacin and tilmicosin, on neutrophil function and hematology parameters were examined. The labeled doses of each drug, or sterile saline, were administered to healthy mixed-breed weaned beef heifers approximately 6 months of age. Neutrophils were isolated from peripheral blood samples at 108 and 60 hours pre-treatment, and 12, 36, and 60 hours post-treatment. The neutrophil function assays performed were: random migration under agarose, Cytochrome C reduction, iodination, *Staphylococcus aureus* ingestion, chemotaxis under agarose, and antibody-independent and antibody-dependent cell-mediated cytotoxicity. The results suggest that at therapeutic drug concentrations, danofloxacin and tilmicosin have little clinically significant effect on bovine neutrophil function.

Introduction

Antimicrobials are relied on to kill or inhibit the growth of bacteria at a site of infection. Recent investigations into the effects of antimicrobials on host cells involved in the immune response have suggested additional roles for certain antibacterial compounds, e.g., modulation of neutrophil function (Labro, 1998). Neutrophils are an arm of the non-specific immune system and contain several mechanisms for bacterial killing. During the normal response to an infectious process, neutrophils marginate, diapedese through the

vascular wall, migrate via chemotaxis to the site of infection, and perform their killing functions. A battery of assays allows the evaluation of many of these steps (Roth, 1993). See Table 1 for an overview of these assays.

The importance of neutrophils in the initial line of defense in bacterial infections cannot be overlooked; however, in some cases an influx of neutrophils may do more harm than good. In the case of bovine pneumonic pasteurellosis, there is evidence to suggest that neutrophil infiltration into the lungs is responsible for a large portion of the lung damage in advanced disease (Slocombe et al., 1985;Breider et al., 1988). Therefore, products that inhibit the response of neutrophils to infections might be advantageous under these circumstances.

The bulk of the investigations into the effects of antimicrobials on neutrophil function have involved in vitro exposure of isolated neutrophils to the antimicrobial, followed by assays of function or morphological examination of the cells (Ziv et al., 1983;Nickerson et al., 1985;Paape & Miller, 1990;Paape et al., 1991;Hoeben et al., 1997a;Hoeben et al., 1997b;Hoeben et al., 1998). The difficulty associated with clinical application of these studies is that the concentration of antimicrobial added in vitro is often much higher than the achievable concentration in the animal, even in tissues where lipid soluble antimicrobials might be expected to accumulate. For example, the lipid-soluble antimicrobial tilmicosin has been demonstrated to accumulate in bovine neutrophils at 40 times the extracellular concentration when the extracellular concentration was 5 μg/ml (Scorneaux & Shryock, 1999). However, the actual maximal serum concentration (C_{max}) of tilmicosin in bovine serum has been reported as 0.873 μg/ml (Modric et al., 1998), suggesting a neutrophil intracellular concentration of approximately 35 μg/ml, which is

considerably lower than the concentrations shown to significantly affect neutrophil function in the in vitro studies (Ziv et al., 1983;Nickerson et al., 1985;Paape & Miller, 1990;Paape et al., 1991;Hoeben et al., 1997a;Hoeben et al., 1997b;Hoeben et al., 1998).

Several studies have evaluated the ability of antimicrobials to modulate bovine neutrophil function, although only the effects of macrolides and fluoroquinolones will be described. Differences in the phagocytosis of radio-labeled *Staphylococcus aureus* by neutrophils were not significant among non-treated and erythromycin-treated neutrophils isolated from bovine milk with erythromycin concentrations of 1000, 500, 10 or 1 μg/ml (Ziv et al., 1983;Nickerson et al., 1985), nor among non-treated and quinolone-treated neutrophils at concentrations of 1000, 500 and 10 μg/ml (Paape et al., 1991). To compare in vitro concentrations with those actually achieved in vivo in the serum, it should be noted that maximal serum concentration of danofloxacin in cattle after intramuscular dosing of 5 mg/kg was 0.83 μg/ml (Mann & Frame, 1992). Lung concentrations after 5 mg/kg intramuscularly approached 5 μg/g (Mann & Frame, 1992). The extrapolated initial serum concentration of erythromycin after intravenous administration of 15 mg/kg in calves was 23.4 μg/ml (Burrows, 1985).

The effects of antimicrobials on phagocytosis of fluorescein-labeled *E. coli* and on respiratory burst activity on bovine neutrophils isolated from peripheral blood were examined by Hoeben et al. (Hoeben et al., 1997b). Erythromycin resulted in a decrease in phagocytosis only at an extracellular concentration of 1000 μg/ml, and danofloxacin at any level had no effect on phagocytosis. Respiratory burst activity was inhibited by danofloxacin at doses of 100 μg/ml and higher and by erythromycin at 1000 μg/ml. Danofloxacin also

decreased myeloperoxidase activity at 1000 μg/ml. Neither erythromycin nor danofloxacin had any effect on neutrophil generation of superoxide anion in that study.

To avoid the concern of applicability of in vitro concentrations to the clinical setting, in this study, we evaluated the ability of the antimicrobials danofloxacin (a fluoroquinolone) and tilmicosin (a macrolide) to modulate neutrophil function in healthy animals. Evaluating therapeutically relevant doses of antimicrobials given to healthy cattle provides a baseline for evaluating neutrophil function in cattle with bovine respiratory disease or other bacterial diseases that are treated with an antimicrobial.

Materials and Methods

The experimental protocol was approved by the Iowa State University Committee on Animal Care. Laboratory procedures were conducted according to Good Laboratory Practices regulations as established by the Food and Drug Administration.

Animals

Thirty-five mixed-breed, weaned beef heifers approximately 6 months of age were leased from a herd in southeastern Iowa. Thirty animals were selected for treatment by excluding the heaviest and lightest animals to achieve uniformity of size. On the day of treatment, the animals weighed from 207-277 kg. The animals had no history of previous treatment with any macrolide or fluoroquinolone antimicrobials.

The animals were permitted an 8-day acclimation period prior to the initial pre-treatment sample collection. During the study, animals were housed outdoors with an open-front pole barn as shelter in a space providing approximately 165 square feet per head. They were fed free choice hay and a grain supplement containing monensin sodium balanced to allow weight gain of approximately 1.5 lbs/head/day. Prior to sample collection pre- and

post-treatment, animals were observed for signs of clinical illness by an investigator masked to treatment.

Treatment

Pre-treatment blood samples for neutrophil isolation were drawn 4 and 2 days prior to treatment with the antimicrobials. A random number from a uniform distribution on the interval zero to one was assigned to each of these 30 animals. The animals were then sorted by this random number and the first 10 assigned to saline treatment, the next 10 to danofloxacin treatment, and the remaining 10 to tilmicosin treatment.

The antimicrobials were administered at 6 p.m. on Day 0, and blood samples were collected for neutrophil isolation 12, 26, and 60 hours after drug administration. These time periods were selected to allow for the maximal effects of the antimicrobials to be demonstrated.

Antimicrobials

Danofloxacin mesylate[ii] (180 mg/ml) was given at a dosage of 6 mg/kg subcutaneously in the left lateral neck. Tilmicosin[iii] (300 mg/ml) was given at a dosage of 10 mg/kg subcutaneously in the left lateral neck. Control animals were treated with 0.9% saline in a volume equal to a dose of danofloxacin subcutaneously in the left lateral neck.

Hematology

Blood samples were collected via jugular venipuncture and placed in tubes containing EDTA[iv]. An automated cell counter[v] was used to measure total and differential leukocyte

[ii] Advocin, Pfizer Inc, New York, New York.
[iii] Micotil, Elanco Animal Health, Indianapolis, Indiana.
[iv] Vacutainer, Becton Dickinson and Co., Franklin Lakes, New Jersey.
[v] Cell-Dyne 3500, Abbott Labs, Abbott Park, Illinois.

count, erythrocyte count, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelet count, and mean platelet volume.

Neutrophil isolation

Blood samples were collected via jugular venipuncture and placed in 50 ml tubes with acid-citrate-dextrose. Samples were centrifuged at 600 x g for 30 minutes. The plasma, buffy coat and top portion of the red cell layer were aspirated from the samples, leaving approximately 10 ml of volume undisturbed in the tube. Red cells were lysed using two volumes of phosphate buffered deionized water. Isotonicity was then restored with one volume of 2.5% saline. Tubes were centrifuged at 200 x g for 10 minutes and the supernatant discarded. The cell pellets were resuspended in 10 ml of phosphate buffered saline and counted. Samples were suspended with Hanks balanced salt solution to a concentration of 5 x 10^7 cells/ml for use in the neutrophil assays.

Neutrophil assays

These assays have been previously described (Roth & Kaeberle, 1981a;Roth & Kaeberle, 1981b;Lukacs et al., 1985;Chiang et al., 1991). Random migration under agarose was performed using a modification of the method of Roth and Kaeberle (Roth & Kaeberle, 1981a). Six holes were punched in agar containing Minimum Essential Medium with Earles salts containing 0.8% agarose, 10% fetal calf serum, and 1% antibiotic-antimycotic solution. Neutrophils were added to the wells, two samples per animal, and the plates were incubated at 39°C in 5% CO2, for 18 hours. Plates were then flooded with 8% glutaraldehyde for at least one hour. After removing the agar, plates were flooded with 0.1% crystal violet for at least 10 minutes in order to stain the cells adhering to the plates. Plates were read on an

inverted microscope using the 10x planar objective by measuring the distance in millimeters radially from the center of the neutrophil well to the farthest point of random migration.

The Cytochrome C reduction assay was performed by incubating opsonized zymosan with Cytochrome C and neutrophils in Hank's balanced salt solution (HBSS), and the optical density of the supernatant at 550 nm was determined. Background values were measured by replacing zymosan with HBSS, and then subtracted from the results obtained with zymosan.

For the iodination assay, opsonized zymosan, NaI, and ^{125}I-Na were pre-warmed in Earle's Balanced Salt Solution at 39°C, and neutrophils were added. The tubes were tumbled for 20 minutes at 39°C, the reaction stopped with cold 10% trichoroacetic acid, and centrifuged for 10 minutes at 1000 g. After discarding the supernatant, the wash was repeated, and radioactivity in the neutrophils was measured in a gamma counter[vi] and reported as counts per minute (CPM). Results were calculated as follows:

$$\frac{(\text{CPM for experimental sample}) - (\text{CPM for blank})}{(\text{CPM for standard sample})} \times (40 \text{ nmol NaI}) \times \frac{(1.0 \times 10^7 \text{ neutrophils})}{(2.5 \times 10^6 \text{ neutrophils})} \times \frac{60 \text{ mins}}{20 \text{ mins}}$$

For the *S. aureus* ingestion assay, heat-killed ^{125}I-labeled *S. aureus*, anti-*S. aureus* antiserum, and 2.5 x 10^6 neutrophils were incubated in Earle's Balanced Salt Solution at 39°C for 10 minutes. Lysostaphin was added, and the tubes incubated for 30 minutes at 39°C. The reaction was stopped with PBS, and the tubes centrifuged at 4C for 10 minutes at 1250 g. After removing the supernatant and repeating the wash procedure, radioactivity associated with the neutrophils was measured in a gamma counter in counts/minute (CPM). Results were calculated as follows:

[vi] Cobra Auto-Gamma, Packard Instrument Co., Downers Grove, Illinois.

$$\text{Percent ingestion} = \frac{(\text{CPM in reaction tube} - \text{CPM in background tube})}{(\text{CPM in standard tube} - \text{CPM in background tube})} \times 100$$

Chemotaxis was evaluated under agarose, using zymosan activated bovine serum as the chemoattractant. After measuring the distance migrated toward Medium 199 (random migration) and the distance migrated toward the activated serum (chemotaxis), a chemotaxis index was generated by dividing chemotaxis by random migration.

The antibody-dependent and antibody-independent cell-mediated cytotoxicity assay was performed as follows: radiolabeled (^{51}Cr) chicken erythrocytes (cRBC's) in Medium 199 were incubated for 2 hours at 37°C in 5% CO_2 with neutrophils in the presence or absence of anti-cRBC antibody. Triton X, neutrophil only, and antibody controls were included. Each animal was represented in quadruplicate. Supernatant was filtered out using a Skatron harvester apparatus. Samples were then placed in a gamma counter and read for 2 minutes. Results were expressed as a percentage of lysis and calculated as follows:

$$\%\ \text{lysis} = \frac{(\text{Counts per minute in reaction tube}) - (\text{Counts per minute in background tube})}{(\text{Counts per minute in standard tube}) - (\text{Counts per minute in background tube})} \times 100$$

Statistical Analysis

The following variables were natural log transformed prior to analysis: neutrophil chemotactic index, mean corpuscular volume, lymphocyte absolute count, monocyte absolute count, RDW, mean platelet volume, and mononuclear white blood cell count. The transformation resulted in residuals that were more normally distributed and also stabilized the variance. The least squares means were then back-transformed after analysis. The repeatedly measured neutrophil assay variables were analyzed using a general linear

repeated-measures mixed model.[vii] If a significant difference was detected ($p \leq 0.05$) due to day of study or treatment effect, pairwise comparisons among treatments were made. Statistical significance was defined as $p \leq 0.05$.

Results

A significant effect of day of assay was noted for all neutrophil assays except the neutrophil chemotactic index, including pre-treatment. However, no statistically significant differences were found among treatments on any of the pre- or post-treatment days for the following neutrophil assays: neutrophil random migration, percent *S. aureus* ingestion, iodination, unstimulated Cytochrome C reduction, antibody-independent cytotoxicity, or antibody-dependent cytotoxicity. (See Tables 2-8 and Figures 1-7 for summaries of results.) No statistically significant differences were found among the following blood cell parameters: total erythrocyte count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total lymphocyte count, total monocyte count, total eosinophil count, total basophil count, red cell distribution width, or mean platelet volume.

The only neutrophil function assay exhibiting any differences among treatment groups was Cytochrome C reduction: The mean optical density of the stimulated cytochrome C reduction assay at 60 hours after treatment was lower for tilmicosin than for saline- or danofloxacin-treated animals (Table 6, Figure 5). The values on all other days before and after treatment were not significantly different.

Total white blood cell count was higher for the tilmicosin treatment group as compared to saline or danofloxacin at 12 hours post-treatment; least squares means for

[vii] SAS Open VMS version 6.12 for an alpha, 1998, SAS Institute Inc., Cary, North Carolina.

controls, danofloxacin-treated and tilmicosin-treated animals were 7.78, 8.63, and 11.39 x $10^3/\mu l$ respectively (Table 9, Figure 8). Total white cell count was also higher for the tilmicosin treatment group as compared to saline but not danofloxacin at 36 hours after treatment. At 12 hours post-treatment, absolute neutrophil count was higher in the tilmicosin treatment group as compared to the other two treatments: 3.63, 4.57, and 6.65 x $10^3/\mu l$ for control, danofloxacin- and tilmicosin-treated animals, respectively (Table 10, Figure 9). The neutrophil counts were above the normal limits for the danofloxacin- and tilmicosin-treated animals. No other differences among white blood cell parameters were noted at any other time.

Total hemoglobin (Table 11, Figure 10) and hematocrit (Table 12, Figure 11) were significantly lower in the danofloxacin treatment group at 12 hours as compared to tilmicosin and controls. There were no other differences among erythrocyte parameters at any other time point.

Mean total platelet count was significantly higher in the control group as compared to the other two treatment groups at 4 days pre-treatment, and was higher than the upper normal limit for cattle reported by the Clinical Pathology Laboratory at Iowa State University (848,000 vs. 800,000, respectively) (Table 13, Figure 12). At this time period, the mean platelet counts for danofloxacin and tilmicosin were 477,000 and 583,000 respectively. At 2 days pre-treatment, mean platelet count in the tilmicosin treatment group was significantly lower than the saline group although the counts were all within the normal limits (100,000-800,000). No differences were found among treatment groups at 12, 36 or 60 hours after treatment.

Two animals showed evidence of adverse reactions after treatment with danofloxacin. One animal went into lateral recumbency for several minutes, but then appeared to recover with no ill effects. The other animal appeared to have an anaphylactoid response, was treated appropriately and was removed from the study.

Discussion

In this study, circulating neutrophils were collected from calves treated with danofloxacin and tilmicosin to evaluate the effect of therapeutic concentrations of these antimicrobials on neutrophil function. In addition, hematology was performed to evaluate the effect of these two antimicrobials on blood cell counts.

A large day-to-day variation when performing these assays is expected due to assay variability (Roth, 1993). For the majority of assays, no significant differences were noted among treatment groups within days. This is in contrast to results obtained in *in vitro* studies using supra-therapeutic concentrations of antimicrobials as discussed above.

Among the neutrophil assays, the only significant difference among treatment groups was in one assay (Cytochrome C reduction) at one blood collection time (60 hours after treatment) when the tilmicosin treatment group exhibited a reduction in superoxide anion production. This could be explained as a cumulative effect of the tilmicosin: The elimination half-life of tilmicosin is approximately 29 hours (Modric et al., 1998), allowing for a long exposure time. It is possible that the tilmicosin's effects on neutrophil function require extended exposure before changes are noted in neutrophils. However, because there were no other consistent differences among the neutrophil assays, this appears to be an anomaly rather than a clinically significant statistic. It is likely that the significant decrease in Cytochrome C reduction was due to random chance, since it was observed at only one time

period. These results are consistent with the findings in an induced-infection model after treatment with tilmicosin. In that study, no differences were noted in neutrophils from control and tilmicosin-treated animals for phagocytic activity and nitro blue tetrazolium reduction, a measure of oxidative function (Chin et al., 1998), although lung neutrophils rather than peripheral neutrophils were evaluated.

At 12 hours post-treatment, tilmicosin-treated animals had significantly higher white blood cell counts due to a higher neutrophil count. The total white cell counts were within normal limits of the laboratory (4,000-12,000 cells/μl). However, the neutrophil counts were above the normal limits for the danofloxacin- and tilmicosin-treated animals. The difference in neutrophil count was transient, disappearing by 36 hours after treatment, although the increase in total white cell count persisted and did not disappear until 60 hours after treatment. We found no reports of this phenomenon in the literature for either antimicrobial. It did not appear to be related to the stress of handling, since there was no effect on the control animals.

At 12 hours after treatment, hemoglobin and hematocrit were significantly lower in the danofloxacin-treated group, although both remained within normal limits for the laboratory. There have been reports of fluoroquinolones causing hemolysis in human patients: temafloxacin was removed from the market after reports of hemolysis with or without organ failure that were associated with an autoimmunity to erythrocytes (Blum et al., 1994). However, in the present study, the decrease in hemoglobin and hematocrit was transient and was never below normal limits for the laboratory, suggesting another, albeit unknown, mechanism must have been responsible.

The differences among the groups for the pre-treatment platelet counts, although statistically significant, did not appear to have clinical significance, particularly since no differences were noted after treatment. In addition, since platelet maturation takes 4-5 days, and platelet life span is approximately 10 days (Duncan et al., 1994), it seems unlikely that the effects noted are due to anything other than clumping during collection, variation in counting, or splenic contraction or congestion.

An argument could be made that comparing these two drugs, which have very different pharmacokinetic and pharmacodynamic parameters, is difficult. The study was designed to account for these differences as described below. In addition, the drugs were administered in the manner (a single dose) and at the dose they are used clinically, so it seems reasonable to compare them in this fashion.

To account for the pharmacokinetic differences, the times of sample collection were selected to allow for the effects of each antimicrobial to be adequately evaluated. Since tilmicosin phosphate has a much longer elimination half-life (29.4 hours (Modric et al., 1998)) as compared to subcutaneous danofloxacin (4.3 hours for a 5 mg/kg subcutaneous dose using a 2.5% solution (Mann & Frame, 1992)), samples were collected until 60 hours after treatment.

The pharmacokinetics of the 2 drugs are different: although they peak at similar serum concentrations (danofloxacin at 0.83 /ml at 5 mg/kg SC (Mann & Frame, 1992); tilmicosin at 0.87 at 10 mg/kg SC (Modric et al., 1998)), their elimination half-lives are different. Danofloxacin has an elimination half-life of 4.3 hours (Mann & Frame, 1992), whereas tilmicosin has an elimination half-life of 29 hours (Modric et al., 1998). In addition, their pharmacodynamics differ: fluoroquinolones are considered peak- or concentration-

dependent, meaning that efficacy is associated with a high peak, related to a large area under the time-concentration curve, rather than a long duration of serum drug concentration (Drusano et al., 1993). In contrast, the efficacy of macrolides is generally considered to be related to the time serum concentration remains above the minimum inhibitory concentration of the organism (Vogelman et al., 1988). Whether these kinetics and dynamics are applicable to other parameters besides their antimicrobial efficacy remains to be determined.

The results from this study suggest that at therapeutic drug concentrations, danofloxacin and tilmicosin have relatively little effect on the functional abilities of circulating neutrophils in healthy animals.

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Table 1. A description of the function assays performed on neutrophils in this study and their interpretation.

Assay	**Interpretation of assay**
Neutrophil random migration	Increased random migration noted in dexamethasone-treated animals; increased random migration implies decreased ability to adhere to endothelium
Neutrophil chemotaxis	Low chemotactic index suggests inability to migrate toward infection
Staph ingestion	Measures ability of neutrophils to phagocytose bacteria
Cytochrome C reduction	Evaluates production of superoxide anion; decrease in production implies decreased ability to kill bacteria
Iodination	Detects dysfunction in the chain of events involving the hydrogen peroxide- myeloperoxidase system; decrease implies decreased ability to kill bacteria
Antibody-independent cytotoxicity	Measures cytotoxicity in the absence of antibody
Antibody-dependent cytotoxicity	Correlates with ability to kill viral infected cells

Table 2. The distance neutrophils migrated under agarose in mm^2 (neutrophil random migration assay), reported as least square means and standard error. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	81.9 ± 10.7	87.4 ± 11.3	78.2 ± 10.7
2 days pre-treatment	96.3 ± 10.7	109.9 ± 11.8	100.2 ± 10.7
12 hours post-treatment	94.7 ± 10.7	115.3 ± 11.3	120.2 ± 10.7
36 hours post-treatment	102.3 ± 10.7	92.5 ± 11.3	110.1 ± 10.7
60 hours post-treatment	110.6 ± 10.7	86.2 ± 11.3	91.4 ± 10.7

Table 3. Neutrophil chemotaxis reported as chemotactic index with 95% confidence intervals. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control	**Danofloxacin**	**Tilmicosin**
4 days pre-treatment	1.09 (1.02-1.16)	1.12 (1.05-1.19)	1.16 (1.09-1.23)
2 days pre-treatment	1.16 (1.10-1.23)	1.09 (1.02-1.15)	1.15 (1.09-1.21)
12 hours post-treatment	1.09 (1.04-1.14)	1.15 (1.09-1.21)	1.06 (1.01-1.11)
36 hours post-treatment	1.08 (0.97-1.22)	1.12 (0.99-1.26)	1.12 (1.00-1.26)
60 hours post-treatment	1.20 (1.11-1.30)	1.20 (1.10-1.32)	1.12 (1.04-1.22)

Table 4. Percent radiolabeled *S. aureus* ingested by neutrophils (*Staphylococcus* ingestion assay) reported as least-squares means and standard error. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	29.1 ± 1.76	23.3 ± 1.85	22.2 ± 1.76
2 days pre-treatment	32.4 ± 1.76	30.6 ± 1.85	33.4 ± 1.76
12 hours post-treatment	38.2 ± 1.76	38.4 ± 1.96	39.1 ± 1.76
36 hours post-treatment	31.5 ± 1.76	32.5 ± 1.85	30.4 ± 1.76
60 hours post-treatment	45.5 ± 1.76	46.1 ± 1.96	41.8 ± 1.76

Table 5. Percent iodination as measured using opsonized zymosan (iodination assay) reported as least-squares means and standard error; evaluates myeloperoxidase/hydrogen peroxide/halide system of neutrophils. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	40.8 ± 4.75	36.9 ± 5.00	32.1 ± 4.75
2 days pre-treatment	32.8 ± 1.74	33.8 ± 1.92	33.3 ± 1.82
12 hours post-treatment	25.5 ± 2.10	30.1 ± 2.21	31.4 ± 2.10
36 hours post-treatment	23.3 ± 1.81	24.3 ± 1.91	25.0 ± 1.81
60 hours post-treatment	23.0 ± 1.99	24.2 ± 2.09	20.7 ± 2.07

Table 6. Optical density of neutrophils incubated with Cytochrome C and opsonized zymosan (Cytochrome C reduction assay) reported as least-squares means and standard error. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	0.46 ± 0.037	0.45 ± 0.041	0.39 ± 0.037
2 days pre-treatment	0.68 ± 0.037	0.66 ± 0.041	0.61 ± 0.037
12 hours post-treatment	0.44 ± 0.037	0.53 ± 0.039	0.49 ± 0.037
36 hours post-treatment	0.44 ± 0.037	0.45 ± 0.039	0.48 ± 0.037
60 hours post-treatment	0.53 ± 0.037 [a]	0.57 ± 0.039 [b]	0.41 ± 0.038 [a,b]

[a]p=0.02, [b]p=0.005; level of statistical significance for the difference between means with the same superscript.

Table 7. Percent lysis of radiolabeled chicken erythrocytes (cRBC) without anti-cRBC antibody reported as least-squares means and standard error. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	4.02 ± 1.30	3.77 ± 2.06	1.85 ± 0.43
2 days pre-treatment	3.21 ± 1.09	2.01 ± 1.48	0.95 ± 0.39
12 hours post-treatment	1.12 ± 0.66	2.83 ± 1.53	0.17 ± 0.11
36 hours post-treatment	4.58 ± 1.90	4.59 ± 2.62	3.28 ± 2.17
60 hours post-treatment	2.18 ± 1.52	6.21 ± 3.96	5.99 ± 3.77

Table 8. Percent lysis of radiolabeled chicken erythrocytes (cRBC) in the presence of anti-cRBC antibody reported as least-squares means and standard error. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	40.86 ± 3.59	41.03 ± 3.79	33.46 ± 3.59
2 days pre-treatment	31.80 ± 3.59	28.94 ± 3.79	27.88 ± 3.59
12 hours post-treatment	19.12 ± 3.59	27.26 ± 3.79	21.01 ± 3.59
36 hours post-treatment	28.40 ± 3.59	31.34 ± 3.79	33.16 ± 3.59
60 hours post-treatment	25.09 ± 3.59	33.08 ± 3.79	27.48 ± 3.59

Table 9. Total leukocyte count from peripheral blood reported as least-squares means and standard error. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	7.07 ± 0.62	7.72 ± 0.65	7.46 ± 0.62
2 days pre-treatment	8.67 ± 0.62	8.94 ± 0.65	9.02 ± 0.62
12 hours post-treatment	7.78 ± 0.62[a]	8.63 ± 0.65[b]	11.39 ± 0.62[a,b]
36 hours post-treatment	6.83 ± 0.62[a]	7.63 ± 0.65	8.81 ± 0.62[a]
60 hours post-treatment	7.81 ± 0.62	8.52 ± 0.65	8.97 ± 0.62

[a]p=0.0001, [b]p=0.003, [c]p=0.03; level of statistical significance for the difference between means with the same superscript.

Table 10. Total neutrophil count from peripheral blood reported as least-squares means and standard error. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	3.08 ± 0.31	3.37 ± 0.53	2.72 ± 0.50
2 days pre-treatment	3.05 ± 0.31	3.37 ± 0.53	3.08 ± 0.50
12 hours post-treatment	3.63 ± 0.31[a]	4.57 ± 0.53[b]	6.65 ± 0.50[a,b]
36 hours post-treatment	2.86 ± 0.31	3.23 ± 0.53	3.4 ± 0.50
60 hours post-treatment	3.04 ± 0.31	3.46 ± 0.53	3.15 ± 0.50

[a]p=0.0001, [b]p=0.006; level of statistical significance for the difference between means with the same superscript.

Table 11. Hemoglobin concentration from peripheral blood reported as least-squares means and standard error. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	13.07 ± 0.26	12.69 ± 0.28	12.79 ± 0.26
2 days pre-treatment	12.46 ± 0.26	12.36 ± 0.28	12.56 ± 0.26
12 hours post-treatment	12.38 ± 0.26 [a]	11.59 ± 0.28 [a,b]	12.43 ± 0.26 [b]
36 hours post-treatment	12.35 ± 0.26	11.99 ± 0.28	12.25 ± 0.26
60 hours post-treatment	12.12 ± 0.26	11.67 ± 0.28	11.80 ± 0.26

[a]p=0.042, [b]p=0.031; level of statistical significance for the difference between means with the same superscript.

Table 12. Hematocrit of peripheral blood reported as least-squares means and standard error. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	36.65 ± 0.72	34.64 ± 0.76	34.63 ± 0.72
2 days pre-treatment	34.09 ± 0.72	33.64 ± 0.76	34.16 ± 0.72
12 hours post-treatment	34.13 ± 0.72 [a]	31.77 ± 0.76 [a,b]	33.98 ± 0.72 [b]
36 hours post-treatment	33.67 ± 0.72	33.03 ± 0.76	33.62 ± 0.72
60 hours post-treatment	33.1 ± 0.72	31.86 ± 0.76	32.30 ± 0.72

[a]p=0.026, [b]p=0.037; level of statistical significance for the difference between means with the same superscript.

Table 13. Total platelet count reported as least-squares means and standard error. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

	Control ± SEM	**Danofloxacin ± SEM**	**Tilmicosin ± SEM**
4 days pre-treatment	848.7 ± 64.1[a,b]	477.4 ± 67.5[a]	582.9 ± 64.1[b]
2 days pre-treatment	711.0 ± 64.1[c]	545.4 ± 67.5	476.7 ± 64.1[c]
12 hours post-treatment	422.5 ± 64.1	449.3 ± 67.5	365.7 ± 64.1
36 hours post-treatment	479.8 ± 64.1	433.3 ± 67.5	387.5 ± 64.1
60 hours post-treatment	518.2 ± 64.1	422.9 ± 67.5	423.8 ± 64.1

[a]p=0.0001, [b]p=0.004, [c]p=0.011; level of statistical significance for the difference between means with the same superscript.

Figure Legends

Figure 1. The distance neutrophils migrated under agarose in mm^2 (neutrophil random migration assay), reported as least square means with standard error bars. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 2. Neutrophil chemotaxis reported as chemotactic index with 95% confidence intervals. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 3. Percent radiolabeled *S. aureus* ingested by neutrophils (*Staphylococcus* ingestion assay) reported as least-squares means with standard error bars. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 4. Percent iodination as measured using opsonized zymosan (iodination assay) reported as least-squares means with standard error bars; evaluates myeloperoxidase/hydrogen peroxide/halide system of neutrophils. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 5. Optical density of neutrophils incubated with Cytochrome C and opsonized zymosan (Cytochrome C reduction assay) reported as least-squares means with standard

error bars. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline. Statistically significant differences between means are indicated by a ($p=0.02$) and b ($p=0.005$).

Figure 6. Percent lysis of radiolabeled chicken erythrocytes (cRBC) without anti-cRBC antibody reported as least-squares means with standard error bars. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 7. Percent lysis of radiolabeled chicken erythrocytes (cRBC) in the presence of anti-cRBC antibody reported as least-squares means with standard error bars. Neutrophils were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

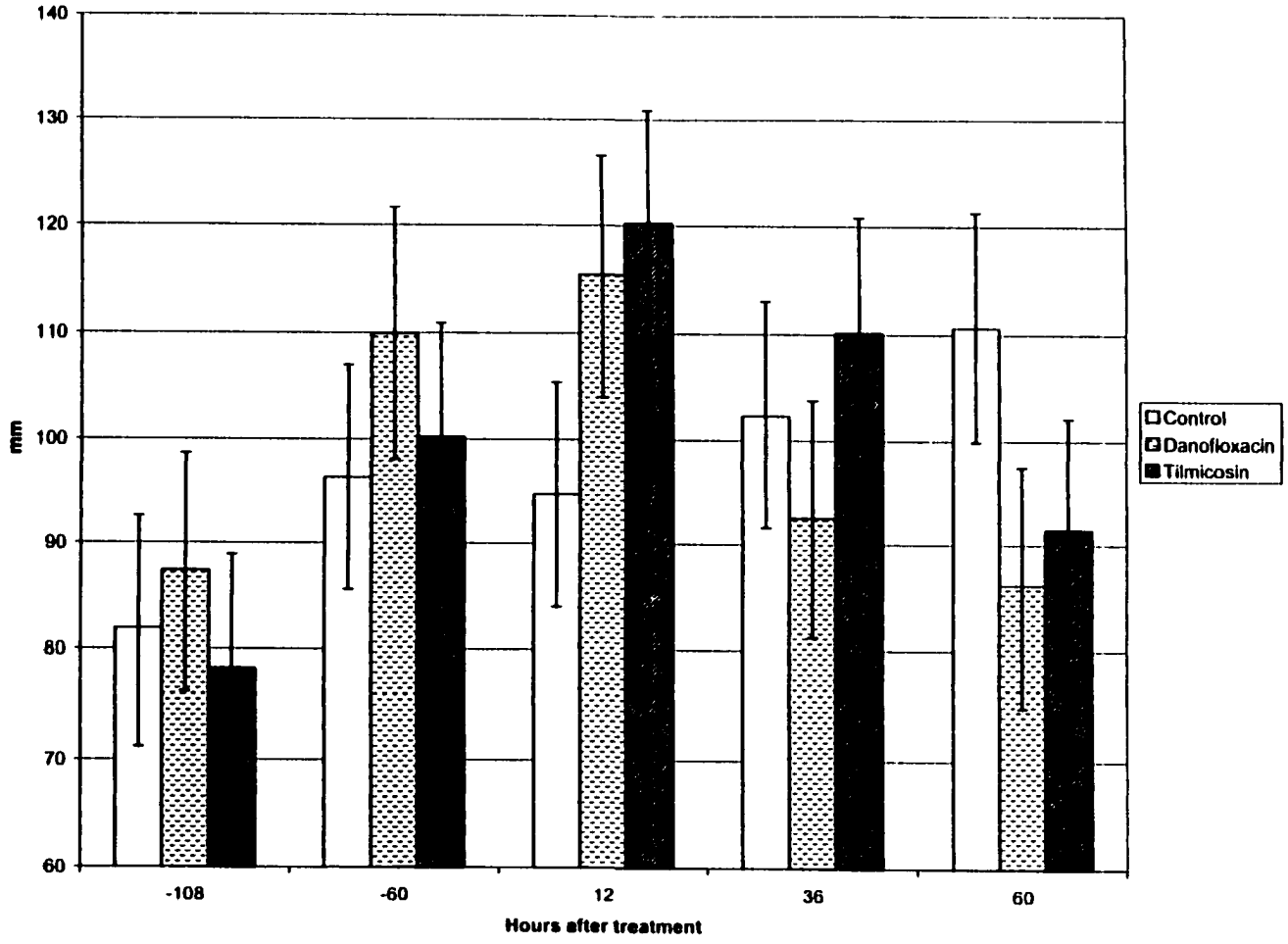
Figure 8. Total leukocyte count from peripheral blood reported as least-squares means with standard error bars. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 9. Total neutrophil count from peripheral blood reported as least-squares means with standard error bars. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 10. Hemoglobin concentration from peripheral blood reported as least-squares means with standard error bars. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 11. Hematocrit of peripheral blood reported as least-squares means with standard error bars. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.

Figure 12. Total platelet count reported as least-squares means with standard error bars. Blood samples were collected 4 and 2 days before and 12, 36 and 60 hours after treatment with danofloxacin, tilmicosin or saline.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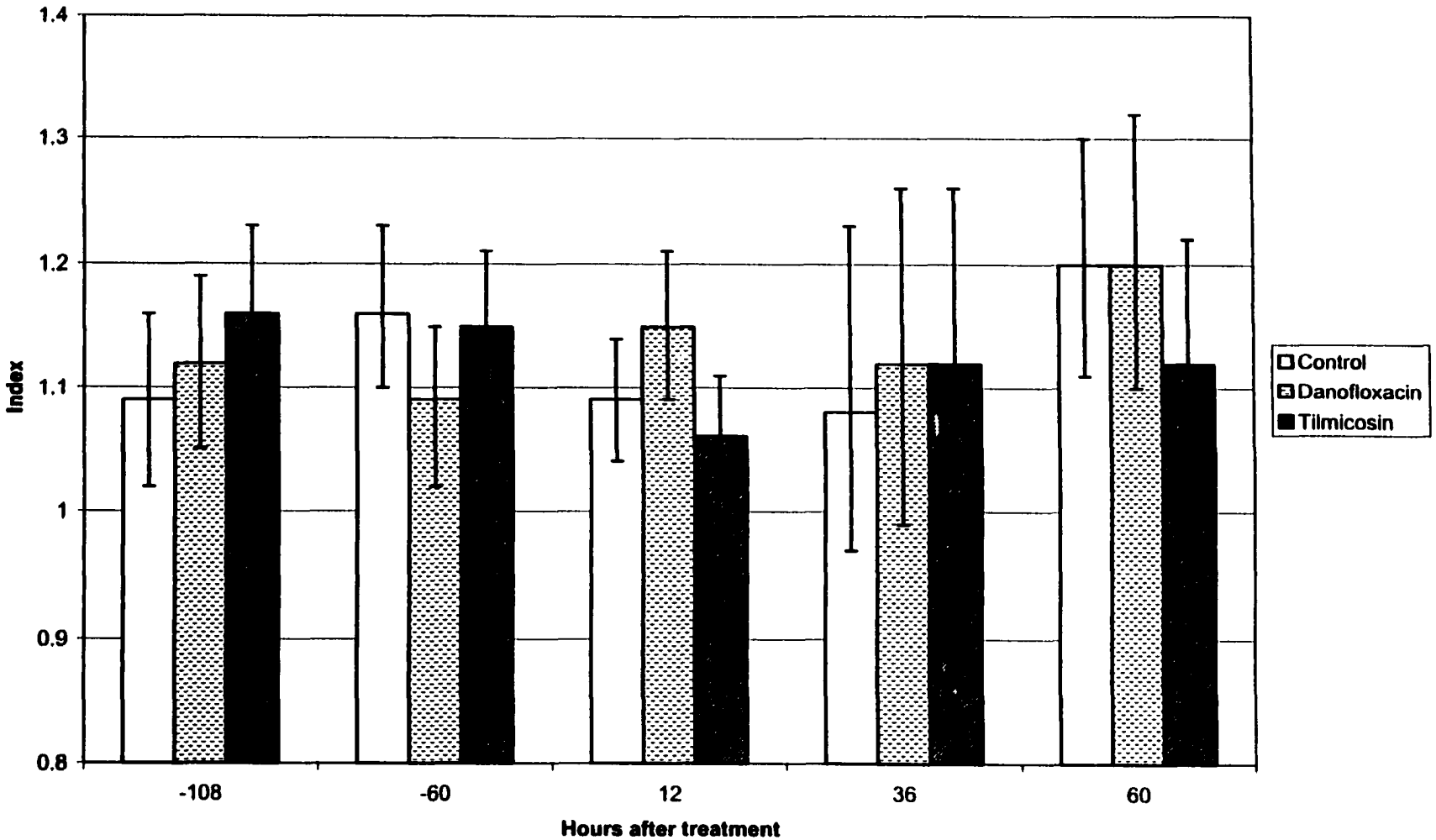
Hours after treatment
Control
Danofloxacin
Tilmicosin

Figure 1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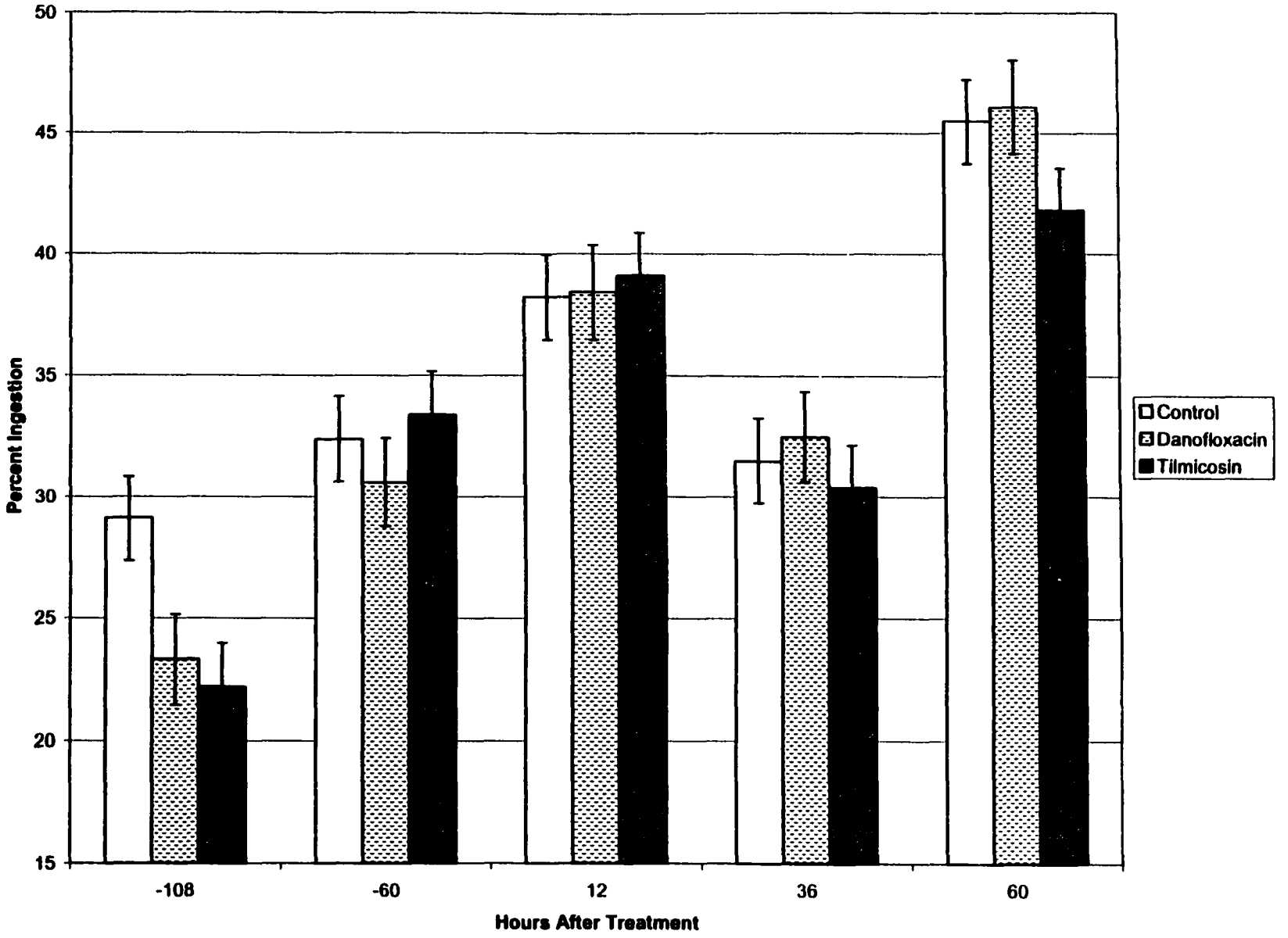
Hours after treatment
Control
Danofloxacin
Tilmicosin

Figure 2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Percent Ingestion
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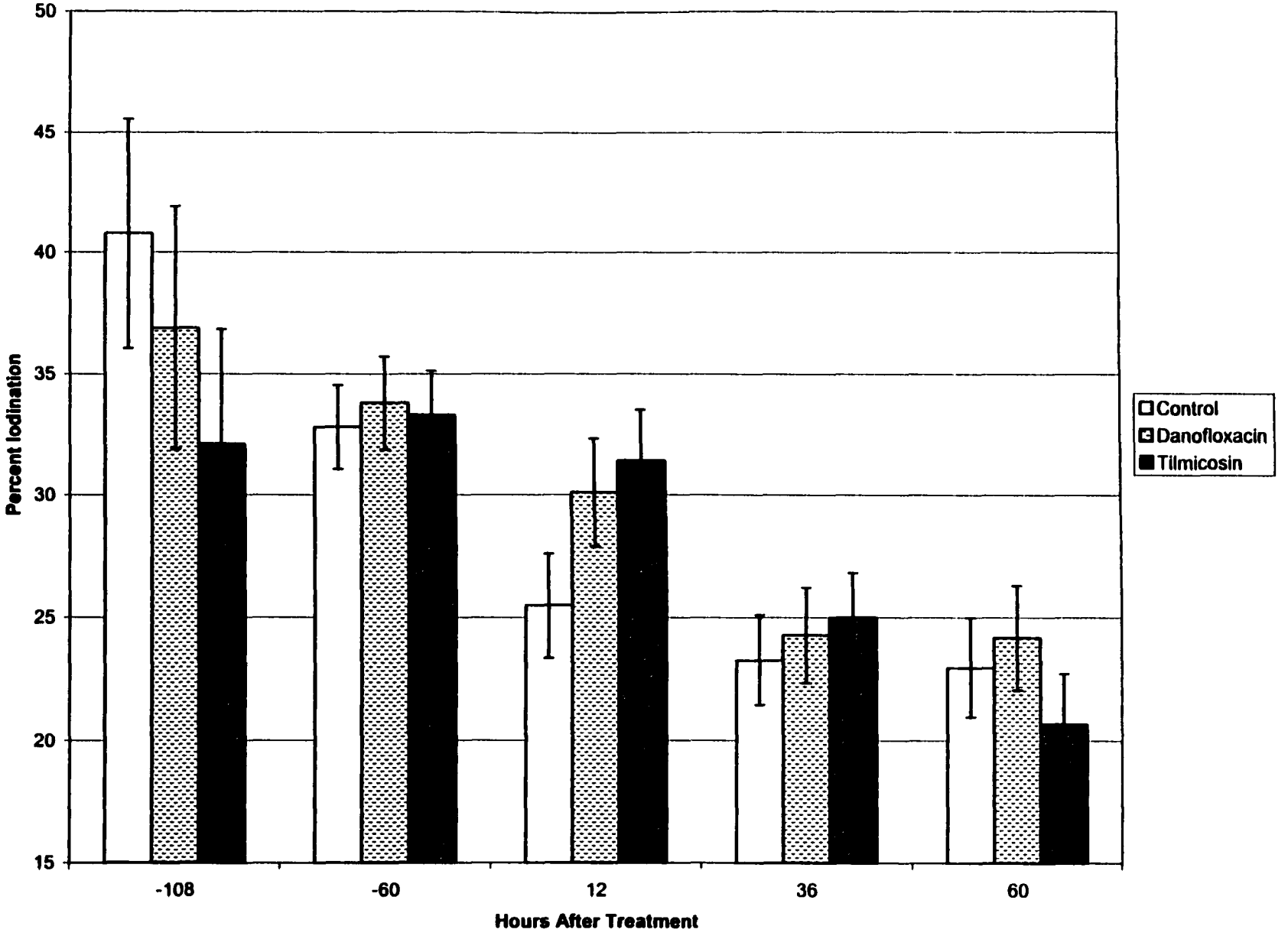
Hours After Treatment
Control
Danofloxacin
Tilmicosin

Figure 3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Percent Iodination
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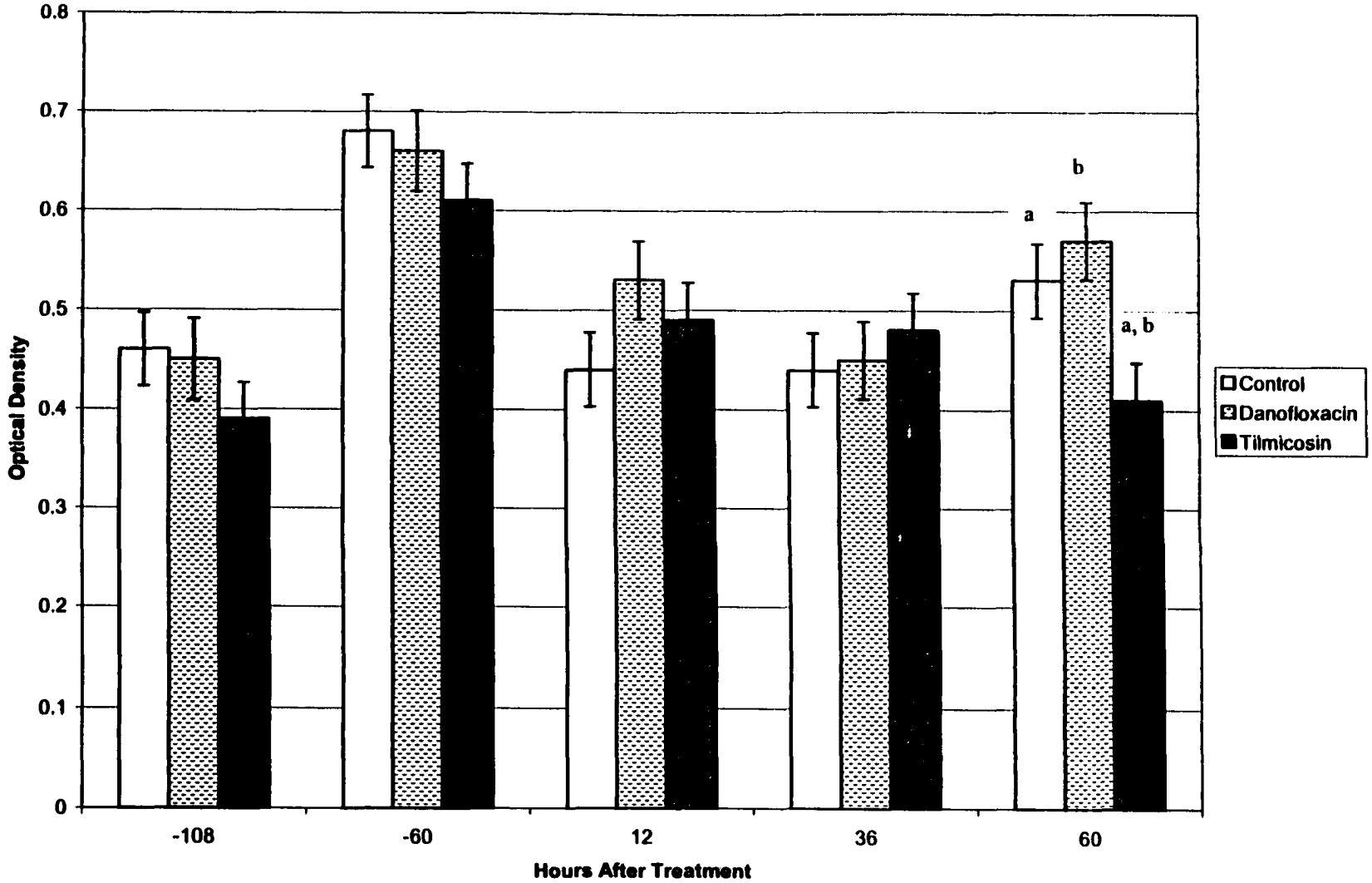
Hours After Treatment
Control
Danofloxacin
Tilmicosin

Figure 4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Optical Density
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36
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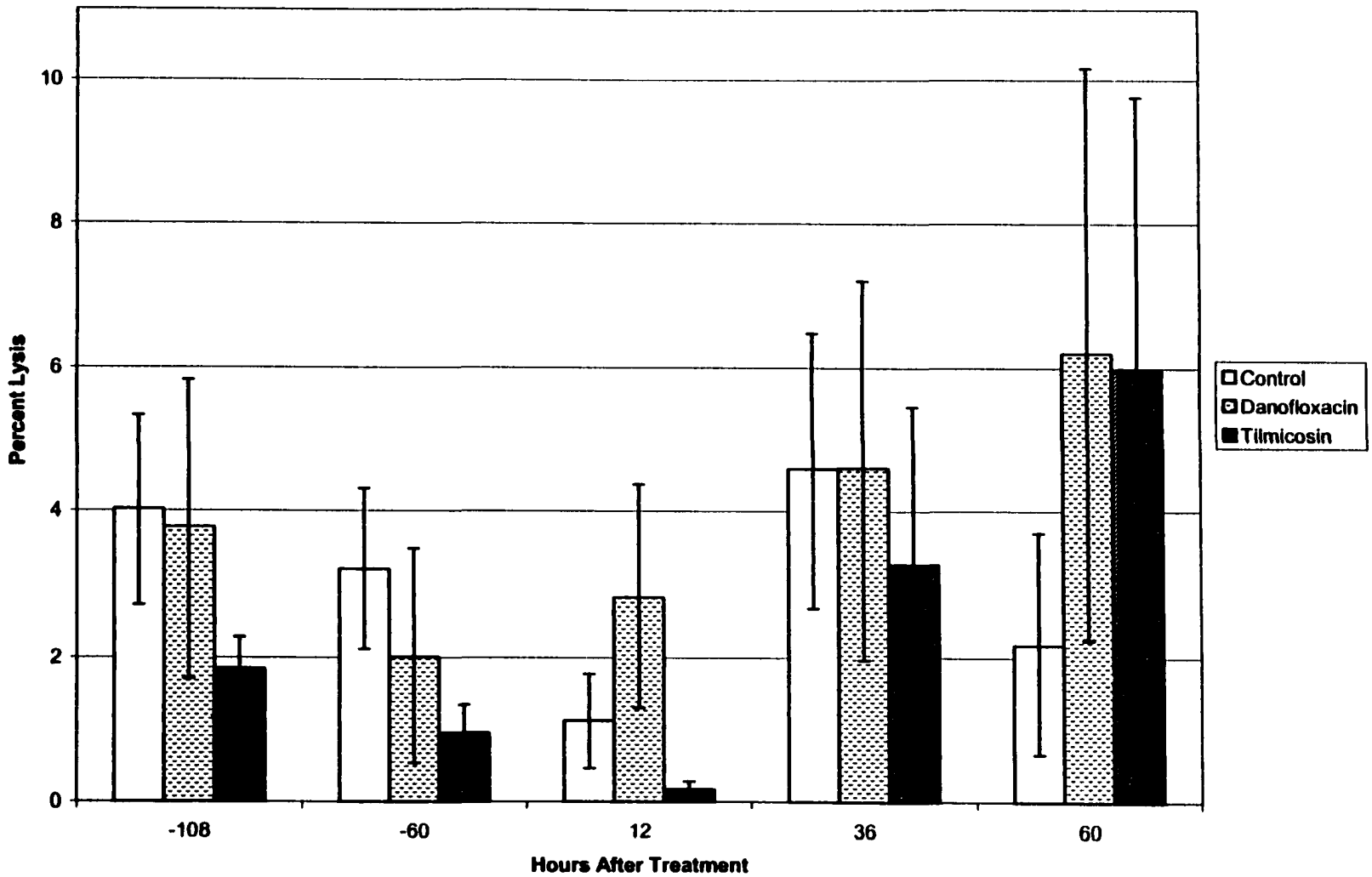
Hours After Treatment
a
b
a, b
Control
Danofloxacin
Tilmicosin

Figure 5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Percent Lysis
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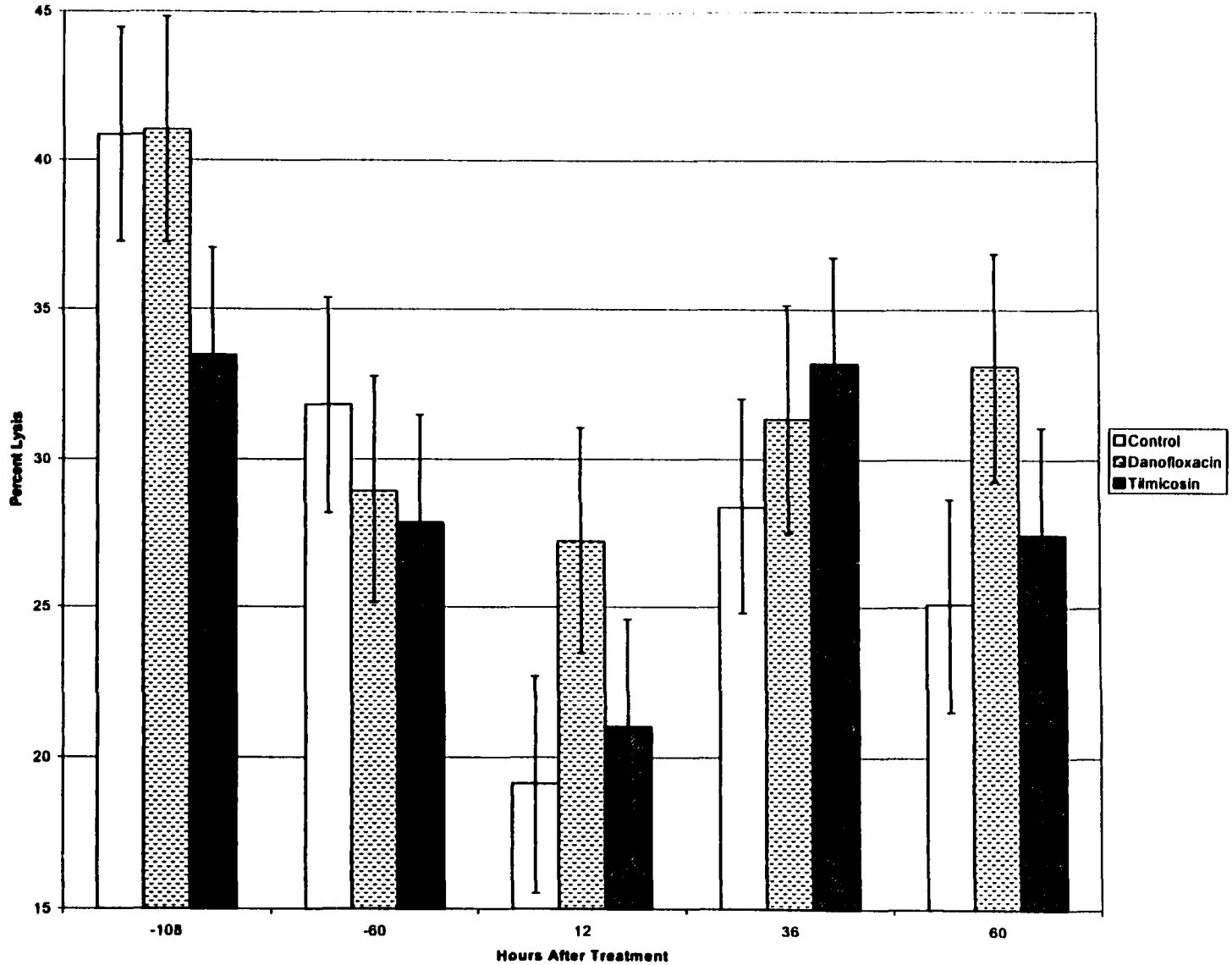
Hours After Treatment
Control
Danofloxacin
Tilmicosin

Figure 6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Percent Lysis
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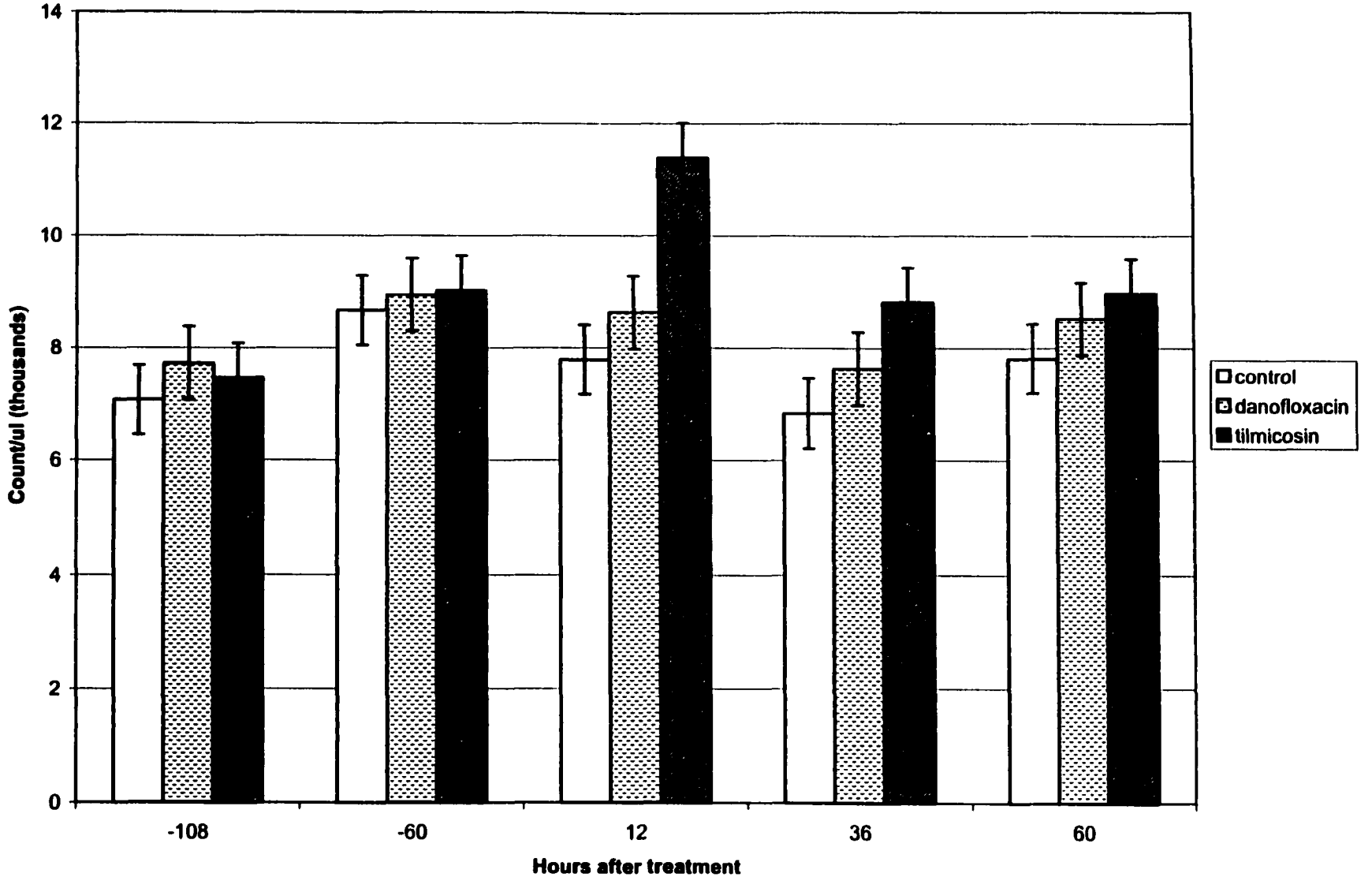
Hours After Treatment
Control
Danofloxacin
Tilmicosin

Figure 7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Count/ul (thousands)
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36
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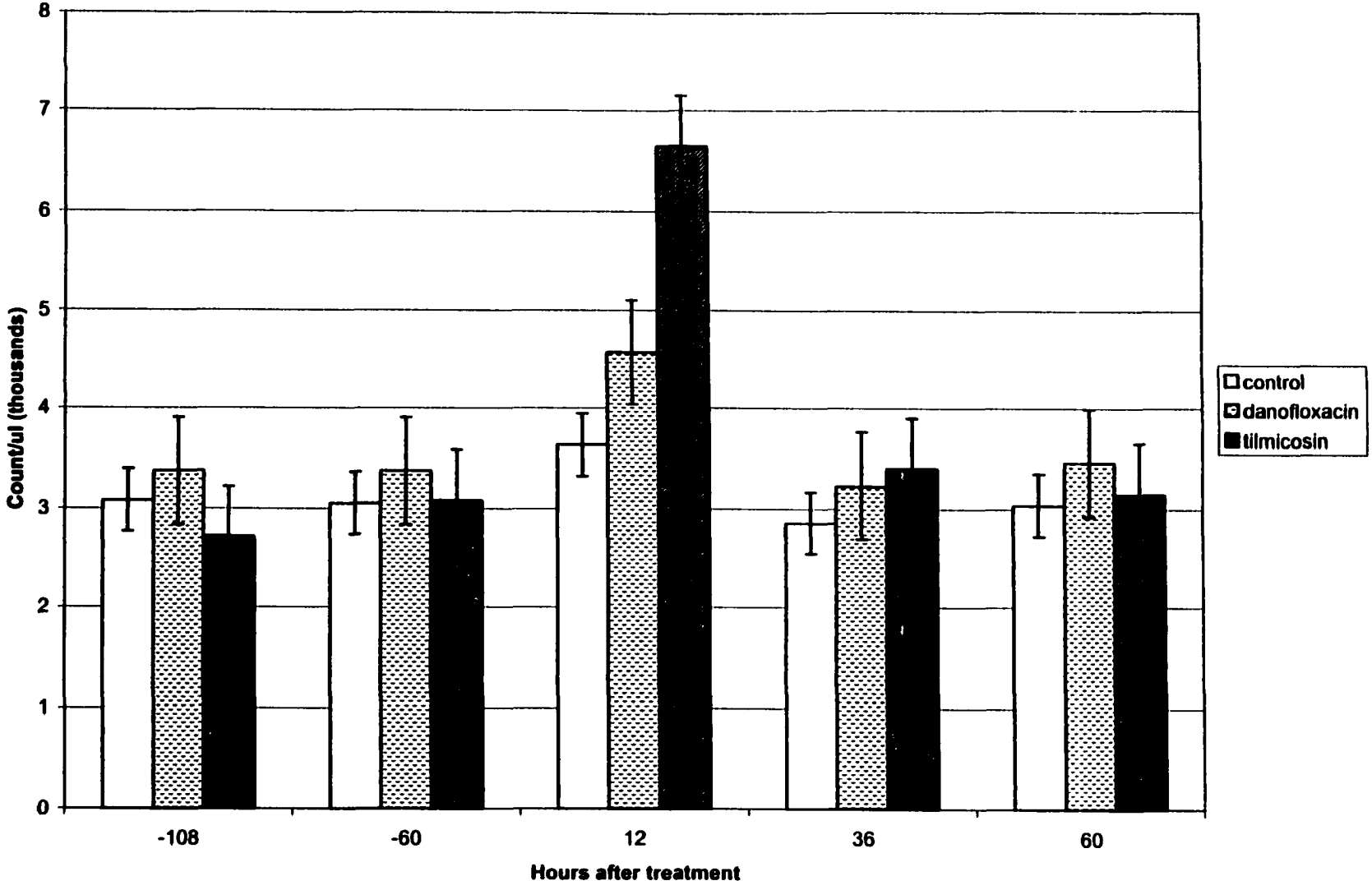
Hours after treatment
control
danofloxacin
tilmicosin

Figure 8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Count/ul (thousands)
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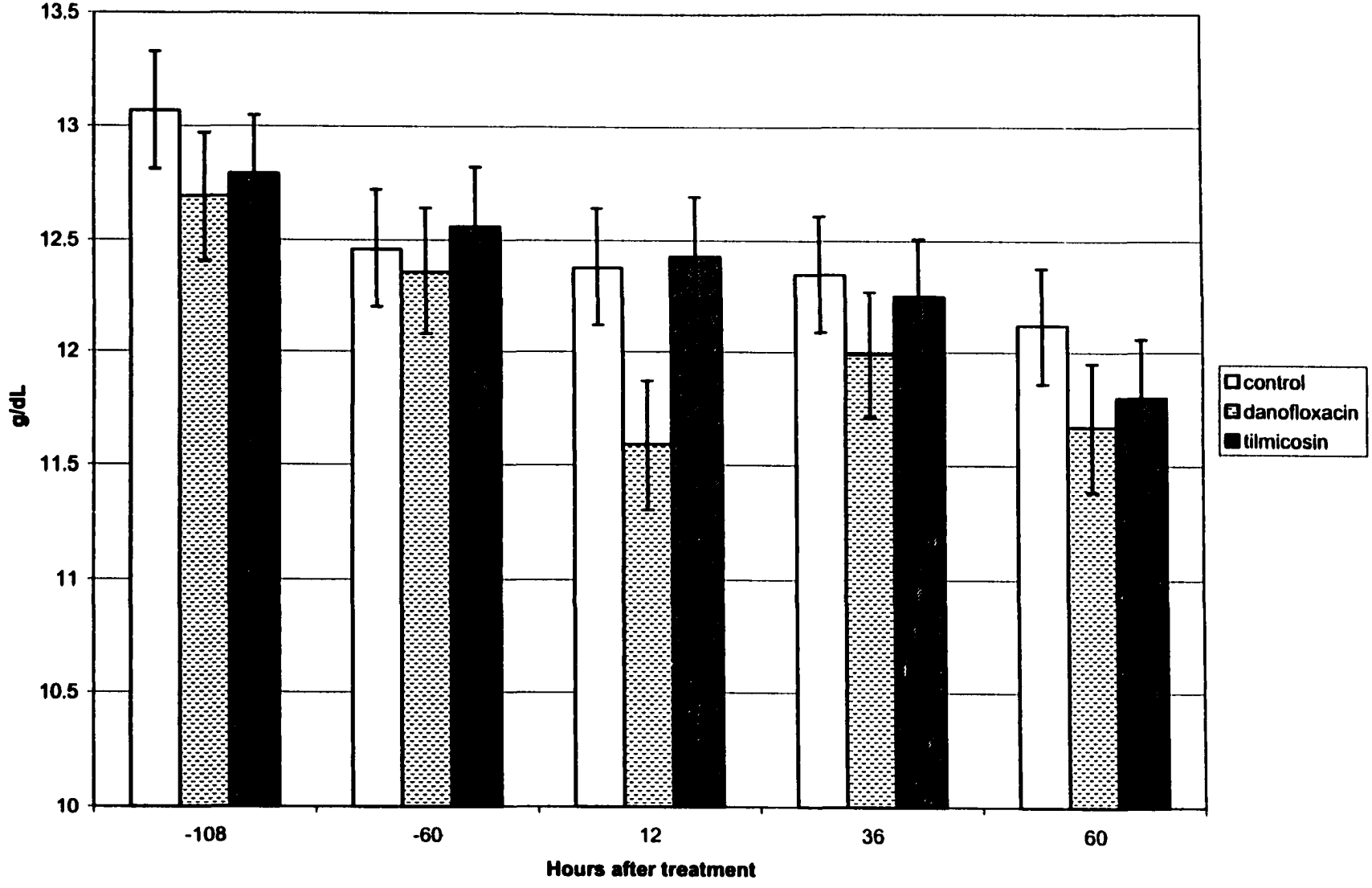
Hours after treatment
control
danofloxacin
tilmicosin

Figure 9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36
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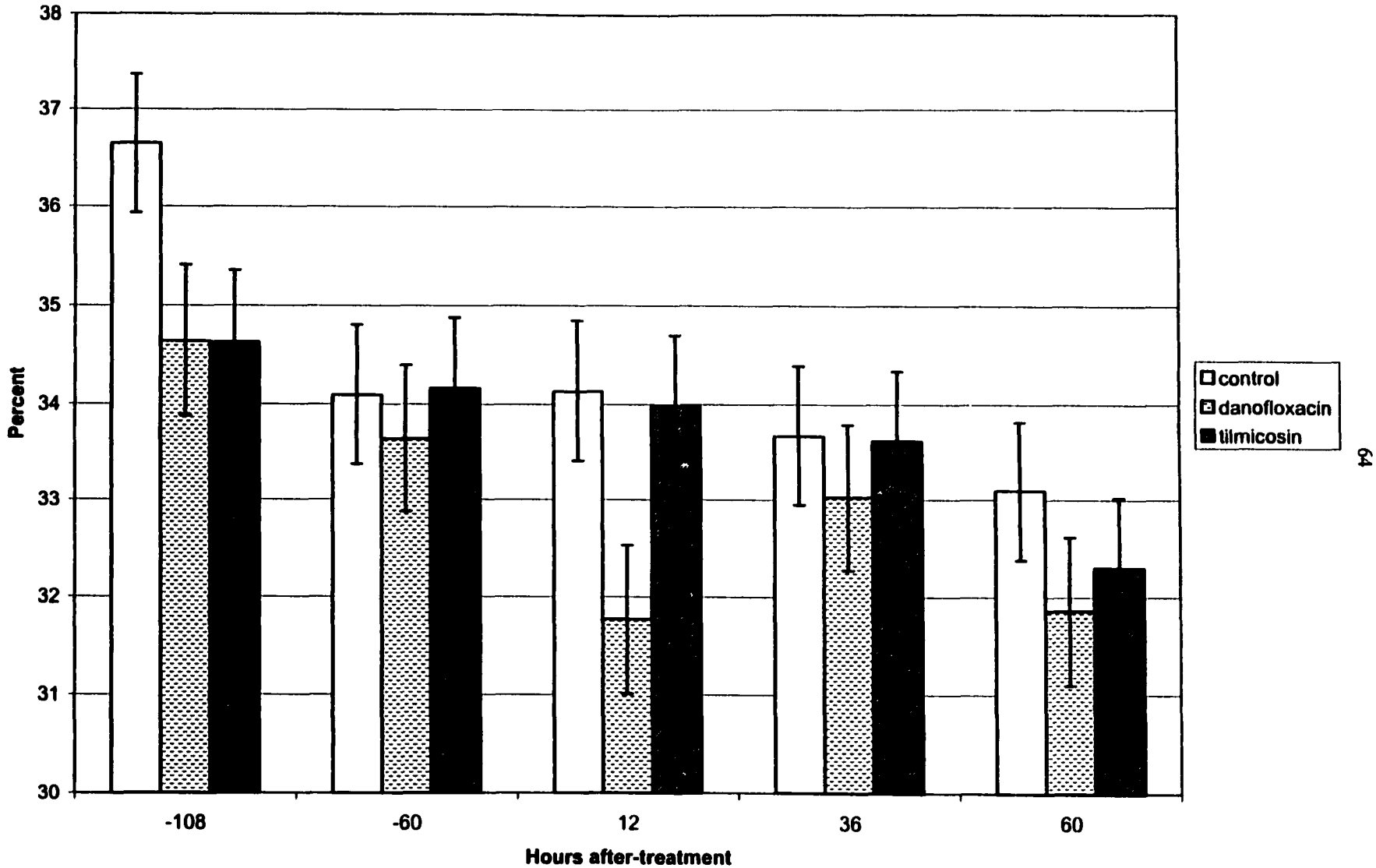
Hours after treatment
control
danofloxacin
tilmicosin

Figure 10


38
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Percent
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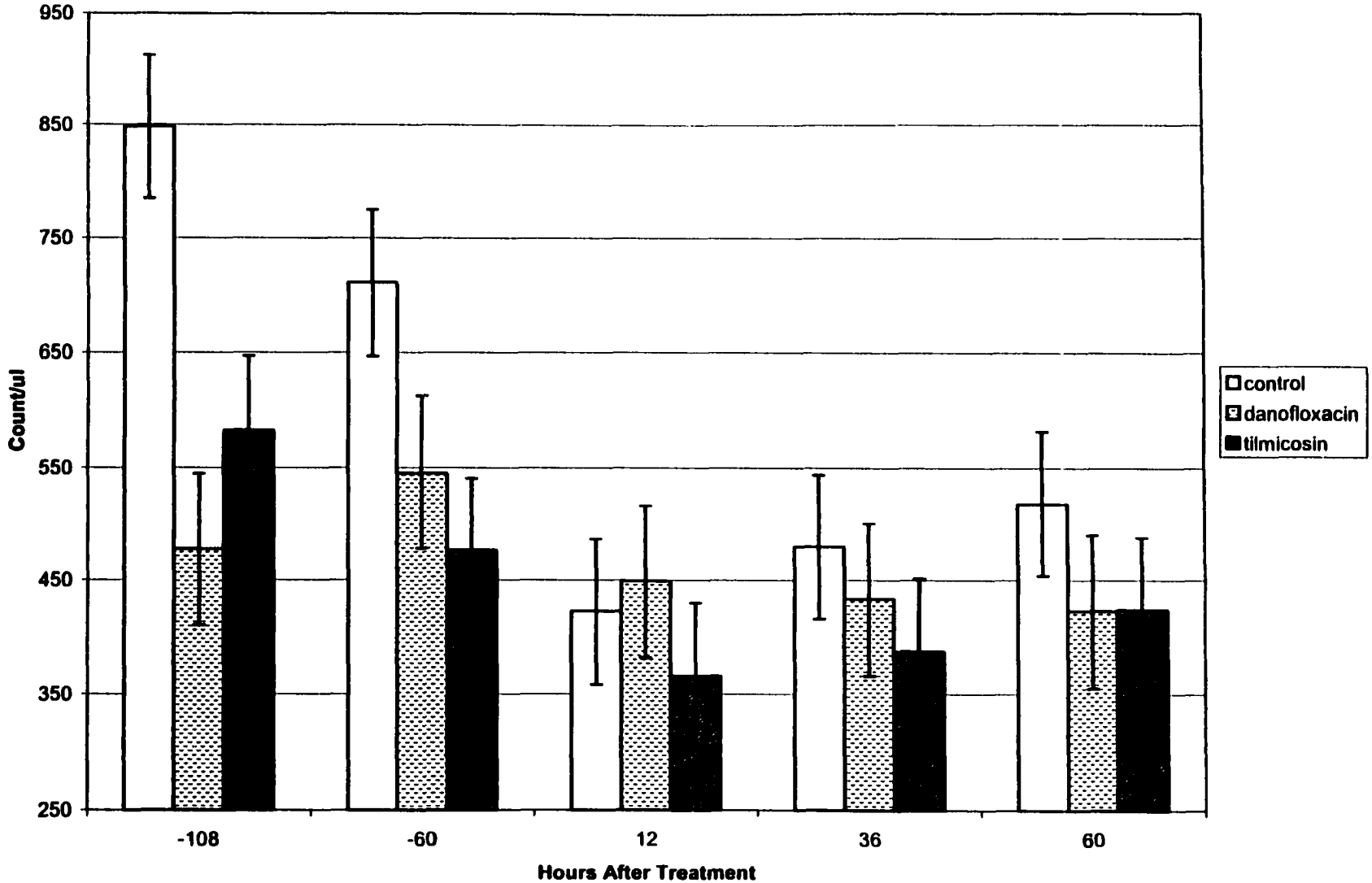
Hours after-treatment
control
danofloxacin
tilmicosin

Figure 11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750
650
550
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Count/ul
-108
-60
12
36
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Hours After Treatment
control
danofloxacin
tilmicosin

Figure 12

CHAPTER 3. DEVELOPMENT OF AN INDUCED *PASTEURELLA* PNEUMONIA MODEL IN WEANED BEEF CALVES

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Virginia R. Fajt, Michael D. Apley, James A. Roth, Kim A. Brogden, Dagmar E. Frank, Terry L. Skogerboe, Valerie Karle, Arthur Dayton

Abstract

Objective

To develop a model of inducing pneumonic pasteurellosis in weaned, pre-conditioned beef calves that consistently produced rectal temperatures of at least 40°C 24 hours after bacterial challenge, a clinical score of ≥1, and measurable lung consolidation; to verify a method of collecting continuous body temperatures via telemetry; and to evaluate the functional ability of lung neutrophils during pneumonia.

Animals

12 weaned Angus-cross beef heifers (172-200 kg) from a single ranch with no recent history of respiratory disease or antimicrobial therapy.

Procedure

Approximately 6 x 10^9 CFU of a log-phase culture of *Pasteurella haemolytica* were instilled intratracheally (via needle puncture) or intrabronchially (via bronchoalveolar lavage catheter). Core body temperatures were continuously monitored via radiotransmitters placed intravaginally in the animals. Bronchoalveolar lavages were performed to collect lung neutrophils for evaluation in neutrophil function assays.

Results

All 8 animals exhibited the desired clinical score and 7 of the 8 the desired rectal temperature at 24 hours after inoculation. Transmitters remained in place for the entire study period. Performance of the lung neutrophil assays was unpredictable; few viable cells could be isolated. Lung consolidation averaged 17.3%, and was histopathologically consistent with pneumonic pasteurellosis.

Conclusions

This model represents a consistent method of producing *Pasteurella* pneumonia in weaned, pre-conditioned beef calves with the desired clinical case characteristics. Lung lavage neutrophil function could not be evaluated, but lung consolidation and body temperature were evaluated with ease.

Clinical Relevance

The model can be used for trials of pharmacotherapeutics to evaluate differences in lung consolidation, core body temperature, and clinical response in weaned pre-conditioned beef calves.

Introduction

Bovine respiratory disease caused by *Pasteurella haemolytica* continues to be one of the most important diseases in the cattle industry worldwide.[1] To study pharmacotherapeutic or immunologic interventions, many methods of inducing experimental pneumonic pasteurellosis have been utilized. This disease has been induced in cattle by instilling fresh live cultures of *Pasteurella haemolytica* into the trachea,[2] into main stem bronchi using an endoscope,[3] into the thoracic cavity percutaneously (transthoracic),[4] or by aerosol nasally.[5] Workers have also used protocols that include physical stressors such as

exercise[6] or cold water[7] and chemical stressors such as acetic acid in the trachea.[8] Many of these models were developed in neonatal dairy calves. In contrast, we were interested in developing a model of inducing pneumonic pasteurellosis in older, weaned, pre-conditioned beef calves without the use of invasive methods such as transthoracic needle puncture and without the necessity of determining *P. haemolytica* antibody titres. In addition, we were interested in developing a model that could be evaluated with a simple clinical scoring scheme rather than a complicated method requiring evaluating multiple clinical parameters.

The use of easily characterized clinical parameters for identification of diseased animals is desirable. Methods for clinical evaluation of calves with bovine pneumonic pasteurellosis have been described that allow for the scoring of animals on a scale from 0 to 4, 0 being normal, and 4 being moribund.[9] In a typical clinical trial of an antimicrobial, animals are eligible for entry into the trial if they exhibit a clinical score of 1, 2, or 3, i.e., ranging from noticeable depression with no apparent signs of weakness to severe depression accompanied by signs of weakness such as altered gait. (Animals are not eligible if they show signs of disease attributable to organ systems other than the respiratory tract.) A rectal temperature of 40°C is commonly recommended as a minimal criterion for inclusion.[9]

An additional requirement in some models is negative or low titres to *P. haemolytica*. A model capable of inducing disease without requiring expensive or time-consuming serology in calves with an unknown history of prior exposure was needed. This would allow increased availability of cattle for experimental pharmaceutical trials.

Accurate, impartial methods for evaluating disease severity are needed to evaluate treatment response. Many studies have recorded rectal temperatures periodically over the course of infection and treatment. However, it is impractical to measure rectal temperatures

more than a few times a day. The use of remote body temperature devices allows close monitoring of changes in body temperature over time. Body temperature radiotelemetry has been utilized in multiple species, and studies in cattle have focused on normal body temperature responses to environmental temperature[10,11] or to estrous cycles[12]. A few reports of the use of radiotelemetry in cattle disease models have also been published, including coliform mastitis.[13] To our knowledge, radiotelemetry of body temperature has not been reported in bovine respiratory disease models. By evaluating body temperature response on an essentially continuous basis during a disease episode and its treatment, differences between treatment groups may be evaluated.

The ability of antimicrobials to modulate the inflammatory process has become an important area of research. In human medicine, the antiinflammatory benefits of many antimicrobials have been reviewed.[14,15] The use of these types of compounds to treat bovine pneumonic pasteurellosis is very attractive, since the inflammation and resultant tissue damage often contributes to the clinical signs of bovine pneumonic pasteurellosis.[16-18] It has been hypothesized that antimicrobials that modulate the neutrophil response may decrease pulmonary damage following infection with *P. haemolytica*. For example, tilmicosin was reported to induce apoptosis of lung neutrophils and therefore to potentially modulate inflammation.[19] To characterize the response of neutrophils as well as attempting to correlate neutrophil activity with clinical response variables, a reliable method of isolating lung neutrophils was needed. In addition, the assays performed needed to be validated for lung neutrophils as they had previously only been performed in our laboratory on neutrophils isolated from blood.

The goals of the study were several. The first goal was to develop a method that consistently produced rectal temperatures of at least 40°C at the time of treatment, with a clinical score of 1 or greater, and measurable lung consolidation in weaned, pre-conditioned beef calves with unknown history of exposure to *P. haemolytica*. Other objectives included the refinement of a method of continuously monitoring body temperature using telemetry, and the evaluation of a method of collecting lung neutrophils and measuring their functional capabilities.

Materials and Methods

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

Animals

Angus-cross heifers weighing an average of 181.3 kg (SD=19.0) on arrival, approximately 6 months of age, 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 were pre-conditioned prior to their arrival at the study site with modified live viral respiratory vaccines; no *Pasteurella* vaccine was administered. Animals that exhibited any clinical signs of disease before *P. haemolytica* inoculation were removed from eligibility for the study and treated as appropriate.

Animals were housed outdoors in concrete-floored pens, one pen per group, with open front sheds as shelter. Area provided per animal was approximately 35 m^2. They were fed free choice hay and a grain supplement balanced to allow weight gain of approximately 1.5 lbs/head/day. Water was provided ad libitum. The grain supplement contained monensin sodium at a rate of approximately 150 mg/head/day.

Twelve animals weighing 172-200 kg were selected for the *P. haemolytica* inoculation procedure as follows: A random number from a uniform distribution on the interval (0,1) was assigned to each eligible animal. The animals were sorted by this random number, and the first 20 were assigned to the procedure. The rest were assigned to be alternate animals in the order in which they were sorted.

Clinical Case Criteria

The clinical scoring system used has been previously described.[9] Briefly, a score of 0 is a normal animal, 1 is noticeable depression without apparent signs of weakness, 2 is marked depression with moderate signs of weakness without significantly altered gait, 3 is severe depression with signs of weakness such as significantly altered gait, and 4 is moribund and unable to rise. Clinical scores were determined just prior to inoculation with *P. haemolytica*, and at 6, 24, 48, 72 and 96 hours after inoculation. The 24-hour time point corresponds to the point at which treatment would be administered in a therapeutic trial.

The successful induction of respiratory disease was defined as a rectal temperature of $\geq$ 40°C and a clinical score of 1 or greater, meaning noticeable to severe depression with or without signs of weakness. Animals that displayed a clinical score of 4 (moribund, unable to rise) were immediately evaluated for euthanasia and were not eligible for the study.

Bacterial Challenge

A frozen (-70°C) stock culture of *Pasteurella haemolytica* A1 strain L101 isolated from a calf with pneumonia and then stored at –70°C was used. After thawing, the culture was streaked on Trypticase Soy Agar plates containing 5% defibrinated sheep blood and incubated overnight at 37°C. The next morning, the growth was transferred to tryptose broth and incubated for 3 hours at 37°C on a magnetic stirrer. Then the broth culture was adjusted

spectrophotometrically to an approximate concentration of 1 x 10^8 colony forming units/ml. Concentrations of bacteria were verified after the inoculation procedure with standard plate counts by removing 1 ml of inoculum, performing 10-fold serial dilutions up to 1x10^6, and plating 0.1 ml of each dilution on 5 blood agar plates. After incubating overnight at 37°C, the number of colony forming units was counted and averaged over the 5 plates.

For the first group of 4 calves (Group 1), 15 cc of culture fluid was administered transtracheally using a 14-gauge needle. For the second group of 4 calves (Group 2), 20 cc of culture fluid was administered transtracheally. For the third group of 4 calves (Group 3), 20 cc of culture fluid was administered to each calf through an equine bronchoalveolar lavage catheter.[a] Briefly, the catheter was passed through the nares into the trachea as far as possible into a caudal lung lobe, then withdrawn approximately 20 cm so that inoculation would occur close to the level of the tracheal bifurcation. Culture fluid was placed in the catheter, followed by approximately 180 ml of air pushed in rapidly with a 60 cc syringe.

Radiotelemetry

Temperatures were monitored using VHF transmitters[b] encased in paraffin. The transmitters broadcast signals in the range of 150.236-151.936 MHz. An antennae and receiver[c] were connected to a personal computer, where the signals were converted to digital pulses by a controller card in a personal computer.[d] The digital pulses were then translated by proprietary software,[e] using calibration charts provided by the company. The inter-pulse interval was proportional to body temperature. The temperatures were automatically stored on the computer hard drive. An uninterruptible power supply was used to prevent data loss during power surges.[f] The antenna was placed on a fence post in the pen, allowing for a maximum of approximately 30 meters from the antenna to any location in the pen.

The transmitters were implanted at least 3 days prior to inoculation of *P. haemolytica* as follows: A low caudal epidural was performed using 3 cc of lidocaine,[g] and then the transmitter was lubricated with sterile jelly[h] and placed intravaginally cranial to the vestibular muscle to ensure retention. The software was programmed to read body temperature every 5 minutes.

Bronchoalveolar Lavage

At 24 and 48 hours after inoculation with *P. haemolytica*, the calves in Groups 2 and 3 were restrained in a squeeze chute, and the head was anchored nose up with two halters. A bronchoalveolar lavage catheter,[a] disinfected between animals with dilute chlorhexidine,[i] was passed through the nasal passages into the trachea, advanced as far as physically possible and wedged into a terminal bronchus. An aliquot of 60 ml of sterile phosphate buffered saline was instilled through in the BAL tube and as much as possible was aspirated back into the syringe. This was performed 2 more times. For each sample, approximately 1 ml was placed in a sterile 10 ml tube for bacterial culture,[j] 1 ml in a EDTA-containing tube for cytology,[j] and the rest in sterile glass tubes for neutrophil isolation.

Samples submitted for cytology were analyzed in an automated cell counter, [k] and the following cells were counted: leukocytes, erythrocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets. Hemoglobin was measured, samples were evaluated visually for transparency and color, and pH and total protein were measured by refractometry.

Neutrophil Isolation

Bronchoalveolar lavage samples were centrifuged at 200 x g for 10 minutes and the supernatant discarded. The cell pellets were resuspended in 10 ml of phosphate buffered

saline and counted. Samples were suspended with Hanks balanced salt solution to a concentration of 5 x 10^7 cells/ml for use in the neutrophil assays. Further purification was attempted with some of the samples by centrifuging over 1.077 specific gravity ficoll-hypaque.[1]

Neutrophil Assays

These assays have been previously described[20-23]. Random migration under agarose was performed using a modification of the method of Roth and Kaeberle. Six holes were punched in agar containing Minimum Essential Medium with Earles salts containing 0.8% agarose, 10% fetal calf serum, and 1% antibiotic-antimycotic solution. Neutrophils were added to the wells, two samples per animal, and the plates were incubated at 39°C in 5% CO_2, for 18 hours. Plates were then flooded with 8% glutaraldehyde for at least one hour. After removing the agar, plates were flooded with 0.1% crystal violet for at least 10 minutes in order to stain the cells adhering to the plates. Plates were read on an inverted microscope using the 10x planar objective by measuring the distance in millimeters radially from the center of the neutrophil well to the farthest point of random migration.

The cytochrome C reduction assay was performed by incubating opsonized zymosan with cytochrome C and neutrophils in Hank's balanced salt solution (HBSS), and the optical density of the supernatant at 550 nm was determined. Background values were measured by replacing zymosan with HBSS, and then subtracted from the results obtained with zymosan.

For the iodination assay, opsonized zymosan, NaI, and ^{125}I-Na were pre-warmed in Earle's Balanced Salt Solution at 39°C, and neutrophils were added. The tubes were tumbled for 20 minutes at 39°C, the reaction stopped with cold 10% trichoroacetic acid, and

centrifuged for 10 minutes at 1000 g. After discarding the supernatant, the wash was repeated, and radioactivity in the neutrophils was measured in a gamma counter[m] and reported as counts per minute (CPM). Results were calculated as follows:

$$\frac{(\text{CPM for experimental sample}) - (\text{CPM for blank})}{(\text{CPM for standard sample})} \times (40 \text{ nmol NaI}) \times \frac{(1.0 \times 10^7 \text{ neutrophils})}{(2.5 \times 10^6 \text{ neutrophils})} \times \frac{60 \text{ mins}}{20 \text{ mins}}$$

For the *S. aureus* ingestion assay, heat-killed ^{125}I-labeled *S. aureus*, anti-*S. aureus* antiserum, and 2.5 x 10^6 neutrophils were incubated in Earle's Balanced Salt Solution at 39°C for 10 minutes. Lysostaphin was added, and the tubes incubated for 30 minutes at 39°C. The reaction was stopped with PBS, and the tubes centrifuged at 4C for 10 minutes at 1250 g. After removing the supernatant and repeating the wash procedure, radioactivity associated with the neutrophils was measured in a gamma counter in counts/minute (CPM). Results were calculated as follows:

$$\text{Percent ingestion} = \frac{(\text{CPM in reaction tube - CPM in background tube})}{(\text{CPM in standard tube - CPM in background tube})} \times 100$$

Chemotaxis was evaluated under agarose, using zymosan activated bovine serum as the chemoattractant. After measuring the distance migrated toward Medium 199 (random migration) and the distance migrated toward the activated serum (chemotaxis), a chemotaxis index was generated by dividing chemotaxis by random migration.

The antibody-dependent and antibody-independent cell-mediated cytotoxicity assay was performed as follows: radiolabeled (^{51}Cr) chicken erythrocytes (cRBC's) in Medium 199 were incubated for 2 hours at 37°C in 5% CO_2 with neutrophils in the presence or absence of anti-cRBC antibody. Triton X, neutrophil only, and antibody controls were included. Each animal was represented in quadruplicate. Supernatant was filtered out using a Skatron

harvester apparatus. Samples were then placed in a gamma counter and read for 2 minutes. Results were expressed as a percentage of lysis and calculated as follows:

$$\% \text{ lysis} = \frac{\text{(Counts per minute in reaction tube)-(Counts per minute in background tube)}}{\text{(Counts per minute in standard tube)-(Counts per minute in background tube)}} \times 100$$

Lung Lesions

Inoculated heifers from Group 2 and 3 were euthanatized with an overdose of barbiturate.[n] At necropsy, 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 less than 10% but greater than 0% consolidation were estimated as 5%. Previously unpublished data[o] derived from bovine lungs obtained from a slaughter house were used to construct the following formula to calculate total lung consolidation:

Total percent lung consolidation = (0.053*cranial segment of left cranial lobe %) + (0.049*caudal segment of left cranial lobe %) + (0.319*left caudal lobe %) + (0.043*accessory lobe %) + (0.352*right caudal lobe %) + (0.061*right middle lobe %) + (0.060*caudal segment of right cranial lobe %) + (0.063*cranial segment of right cranial lobe %)

Areas of consolidation were sampled for histopathology and bacterial culture.

Statistical Analysis

The transmitters were re-calibrated after study completion by the supplier, and correction factors were calculated for each transmitter to account for drift in temperature over the battery life of the transmitters. Because the transmitters are subject to interference, a procedure was established to remove outliers that did not fit with the surrounding data and that were therefore considered anomalous. An overall mean and the standard deviation of the

temperatures over the entire period was calculated. If an observation was greater than 3 standard deviations from the overall mean, the value was removed from the data set. In addition, observations of $\leq 37.5°C$ or $\geq 42.5°C$ were considered outside of the normal physiological range and were removed from the data set. The data were smoothed such that a running average of 10 observations was used to display individual calf temperatures.

The average core body temperature pre-challenge (in °C) for each animal minus 1°C was used as a baseline for that animal. The area under the curve above the baseline for each animal was calculated for each 12-hour interval pre- and post-challenge, and geometric means of the areas under the curve were calculated for Groups 2 and 3. If there were fewer than 10 observations for a given time period, the area under the curve calculation was dropped from the analysis. The arithmetic mean temperature was also calculated for each animal within each 12-hour interval.

Descriptive statistics were calculated for each of the neutrophil assays, for the cytology data, and for the lung consolidation data. A general linear mixed model was used to compare consolidation in the left and right sides of the lungs.

Results

Group 1 calves, to which the challenge inoculum was administered transtracheally at a dose of 15 ml/calf, returned to clinical scores of 0 (no depression) by 48 hours after inoculation, and these animals were not necropsied for lung evaluation. Animals in Group 1 did exhibit a peak in core temperature as measured by the transmitters, but the temperatures were below 40°C by 36 hours after challenge.

The dose of inoculum was increased to 20 ml/calf transtracheally for Group 2. The inoculation procedure was then altered as described above by using a catheter intranasally to

administer the bacterial culture for Group 3. This method was selected due to ease and speed of inoculation, and to attempt to place more inoculum into the bronchi rather than the trachea. At 24 hours, all 8 calves (Groups 2 and 3) that received 20 ml of inoculum exhibited a clinical score of 1 or greater (Figures 1 and 2), and 7 of the 8 calves had a rectal temperature of 40°C or greater.

The radiotransmitters remained in place throughout the experiment (a week or longer). No surgical fixation or other device to prevent expulsion was required. After "no signals" and outliers (data points that appeared to be physiologically improbable) were removed, an average of 361 data points remained for analysis for each calf in Group 2, and 1051 data points for Group 3.

Bronchoalveolar lavages were performed on Group 2 and 3 calves at 24 and 48 hours after inoculation. For 7 of the 8 calves sampled, most of the 180 ml of solution instilled into the lungs was recovered. Cytology results are shown in Table 1; actual neutrophil counts were only obtained on the 24-hour samples. The 48-hour lavages contained very low numbers of neutrophils. Bacterial culture of all the samples from 24 and 48 hours post-inoculation yielded no growth of *Pasteurella haemolytica*, although low to moderate numbers of contaminants such as *Streptomyces* sp., *Pseudomonas* sp. and *Staphylococcus epidermidis* were isolated from all but 2 animals.

Neutrophils were isolated from the lavage fluid from 3 of the 8 calves at 24 hours and from 5 of the 8 calves at 48 hours after challenge. From the 3 calves at 24 hours, 2 of the 3 samples contained mostly mononuclear cells rather than neutrophils. The neutrophils tended to clump and were difficult to isolate even after filtering through a density gradient.[1] There were not enough neutrophils from the 8 successful bronchoalveolar lavage samples to

perform all of the assays, so only certain assays were performed on each sample (see Table 2 for number of assays performed). Even with sufficient sample, the neutrophils performed poorly in the majority of the assays (data not shown). For example, the neutrophil chemotaxis assays and the random migration assays produced no results, since no neutrophils migrated out of the wells.

An estimation of percent consolidation by lung lobe was made visually and by palpation, and the mean percentage by lobe is displayed in Table 3, along with the proportion of the total consolidation contributed by each lobe. Mean total lung consolidation was 17.3%, with a range of 5.0-37.5%. The left lobes contributed 12.1% to the total, and the right lobes contributed 75.1%, which is statistically significant. All calves had some degree of consolidation in the accessory lobe. Grossly, the lung lesions resembled fibrinous bronchopneumonia. Microscopic examination of consolidated areas revealed severe subacute fibrinosuppurative and necrotizing pleuropneumonia consistent with pneumonic pasteurellosis. Bacterial culture of consolidated areas resulted in pure cultures of *P. haemolytica* in 7 of the 8 samples, and a mixed culture of *P. haemolytica* and *P. multocida* in 1 sample.

Discussion

Eight calves with unknown prior exposure to *P. haemolytica* inoculated intratracheally or intrabronchially with approximately 2.0×10^9 cfu of *P. haemolytica* exhibited a clinical score of ≥ 1, and 7 of the 8 developed rectal temperatures of $\geq 40°C$ at 24 hours post-inoculation. The resultant proportion of animals exhibiting the criteria established for entrance into a respiratory disease pharmacotherapeutic trial suggests this inoculation procedure would be acceptable for inducing disease in large numbers of animals. When

using relatively expensive animals such as weaned beef calves, this is an important consideration in choosing a model. The intrabronchial method of inoculation was much easier and faster to perform than the intratracheal in addition to being less invasive than transthoracic. The considerable skin and fat in the throat latch region in these calves made isolating the trachea for injection of the inoculum cumbersome and difficult.

Body temperature radiotransmitters were used successfully and remained in place for the duration of the disease episode, without the use of surgical or physical retention devices. This makes the use of heifers attractive for these types of trials, since surgical implantation of the transmitters is required in steers. The methods used for the descriptive statistics are attractive for comparing treatment modalities in disease episodes. Calculating the area under the curve using a baseline for each individual animal removes the variability of normal core temperatures among animals and allows for statistical comparisons among time periods. The use of average temperatures instead of area under the curve would not characterize the fluctuations in temperature as well, and high peaks or troughs would tend to be underrepresented. Additionally, using the area under the curve helps remove the variability in temperature among animals, since the absolute temperature of the animal is not part of the equation but rather the relative change in temperature around baseline.

The cytology of bronchoalveolar lavages collected resembled the cytology of normal lungs, rather than pneumonic lungs. The samples contained an average of 327 leukocytes/μl and 295 leukocytes/μl at 24 and 48 hours respectively, as compared to 1312-1615 leukocytes/μl in one study of pneumonic calves.[24] The percentage of neutrophils in our study more closely resembled that of Paulson et al. for Group 2 but not for Group 3. In Group 3, neutrophils were 16% of the leukocytes, similar to pre-challenge in the previously mentioned

study where neutrophils were 12% of the total leukocytes. One possible explanation for the difference in our study was the collection procedure. The bronchoalveolar lavage catheter was lodged in a caudal lung lobe before instilling the collection medium. If it lodged in the left caudal lobe, where gross exam at necropsy revealed a mean of 2% consolidation, neutrophil exudation and harvest would be expected to be minimal. This explanation is consistent with the inability to isolate *P. haemolytica* on bacterial culture. In addition, this explanation fits with the lung consolidation data, which suggest that more inoculum reached the right side than the left side of the lung, since 75% of the total consolidation was attributable to the right lung lobes.

The results from the assays on the bronchoalveolar lavage neutrophils were not consistent with findings from the same laboratory using the same assays on blood neutrophils. For example, in a study revealing reduced neutrophil function in cattle persistently infected with bovine viral diarrhea virus, even in the low performing groups, Cytochrome C reduction was 0.74 (vs. 0.16 in our study), iodination was 25 (vs. 0.25-5.65 in our study), and antibody-dependent cytotoxicity was 48% (vs. 13.5-22.4 in our study).[25] Neutrophils failed to migrate in our study in the random migration and chemotaxis assays, suggesting non-viable cells. The low performance of the neutrophils in the function assays along with the low yield of neutrophils from the bronchoalveolar lavages suggest that lung neutrophils are not a good choice for evaluating response to infection, antimicrobial therapy or both. This is in contrast to the findings of Chin et al.,[19] who were able to isolate neutrophils from the lungs of infected calves. However, the assays they selected did not necessarily characterize the viability of the neutrophils. Viable neutrophils are required to perform in the assays used in the present study to evaluate migration, phagocytosis and

oxidative functions. Circulating neutrophils are the pool from which cells are recruited to sites of infection, so the collection of neutrophils from the blood is an attractive alternative. In addition, by collecting peripheral blood neutrophils, potential effects of the *P. haemolytica* itself on neutrophils in the lungs are minimized, since *P. haemolytica* will affect neutrophils by inducing apoptosis,[26] causing cell membrane damage,[27] and decreasing chemiluminescence (a measure of the respiratory burst used to produce oxygen radicals for bacterial killing).[28]

Gross and histologic examination of the lungs at necropsy revealed lesions consistent with naturally-occurring subacute pneumonic pasteurellosis, which concurred with the bacterial culture of the consolidated regions. The percentage lung consolidation found in the present study was consistent with previous work[29,30] and provides another parameter for comparative purposes. In this study, it appeared that more inoculum reached the right lung lobes than the left, even in the calves inoculated intratracheally ($p=0.0001$). Interestingly, only 1 calf in the intrabronchial group had any lesions in the right cranial lobe, suggesting that the bronchoalveolar lavage catheter was in fact caudal to the tracheal bronchus. The resultant lesions, however, were consistent with the natural presentation of pneumonic pasteurellosis.

In summary, we developed a simple method for reliably inducing *Pasteurella* pneumonia in weaned beef calves via bronchoalveolar lavage catheter. The inoculation resulted in greater than 80% of calves developing clinical scores of ≥ 1, rectal temperatures of $\geq 40°C$, and measurable lung consolidation from which *Pasteurella haemolytica* could be cultured. It was determined that virtually continuous measurement of core body temperature and subsequent statistical analysis of the disease episode via temperature was possible in

heifer calves without surgical placement of transmitters. It was also determined that lung neutrophils collected via bronchoalveolar lavage were not sufficiently viable in this model for evaluation of pharmacotherapeutic interventions.

Footnotes

[a]Bivona, Inc., Gary, Indiana

[b]Minimitter® Company, Sunriver, Oregon

[c]TR-2 receiver, Telonics, Mesa, Arizona

[d]Gateway 386 personal computer, North Sioux City, South Dakota

[e]Datacol5® Large Animal Monitoring Software, Minimitter Co., Sunriver, Oregon

[f]American Power Conversion Corp., Back-UPS Pro 420, West Kingston, Rhode Island

[g]Lidocaine 2% Injectable, Abbott Laboratories, North Chicago, Illinois.

[h]K-Y® Lubricating Jelly, Johnson&Johnson Medical Inc., Arlington, Texas

[i]Nolvasan® Solution, Fort Dodge Animal Health, Fort Dodge, Iowa

[j]Vacutainer, Becton Dickinson and Co., Franklin Lakes, New Jersey

[k]Cell-Dyne 3500, Abbott Labs, Abbott Park, Illinois

[l]Ficoll, Sigma, St. Louis, Missouri

[m]Cobra Auto-Gamma, Packard Instrument Co., Downers Grove, Illinois

[n]Beuthanasia®-D Special, Schering-Plough Animal Health, Union, New Jersey

[o]Personal communication, Terry Skogerboe, Pfizer Animal Health, 1998

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Figure Legends

Figure 1. Clinical scores observed for Group 2 calves (n=4) after inoculation (scores are on a scale from 0-4, with 0 being normal and 4 being moribund; see text for complete explanation).

Figure 2. Clinical scores observed for Group 3 calves (n=4) after inoculation (scores are on a scale from 0-4, with 0 being normal and 4 being moribund; see text for complete explanation).

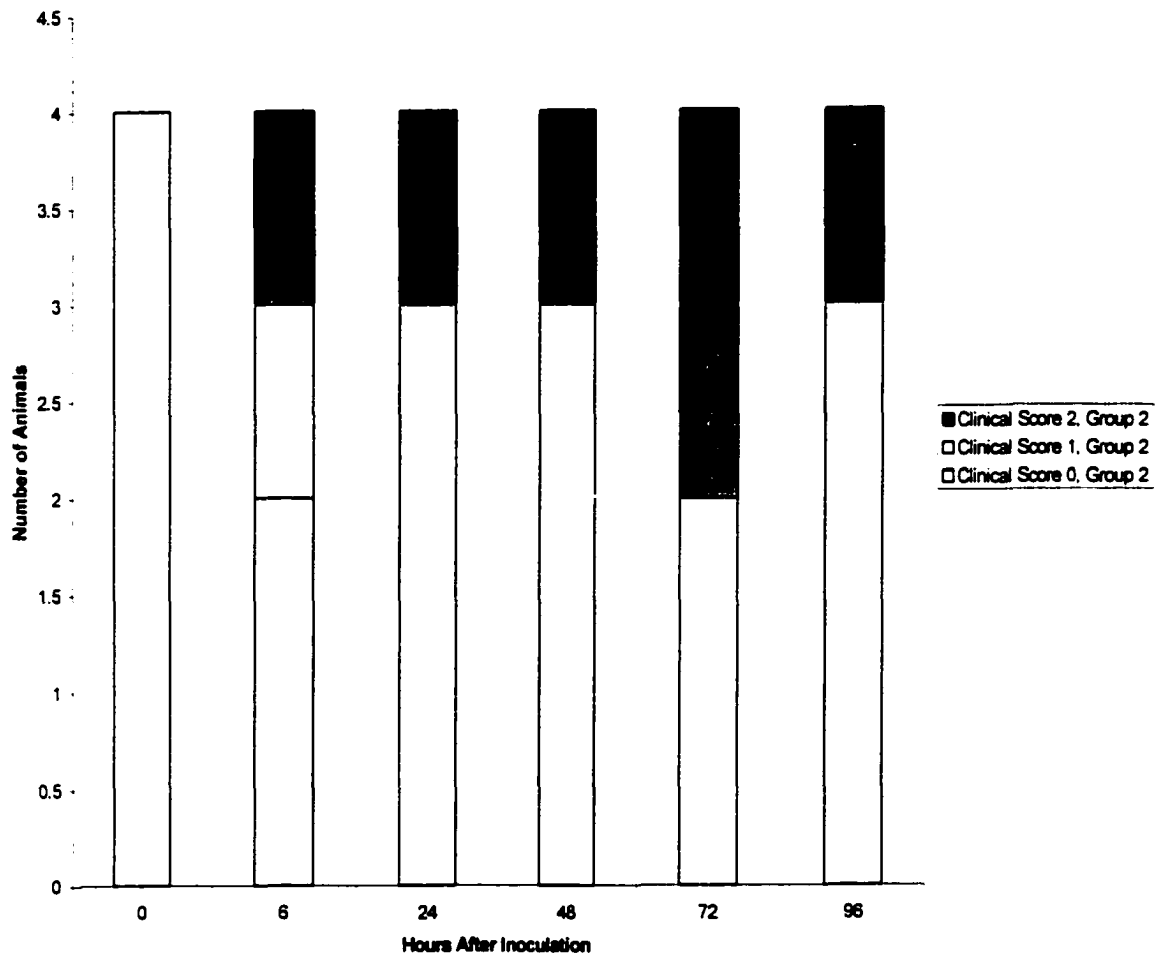
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Number of Animals
0
6
24
48
72
96
Hours After Inoculation
Clinical Score 2, Group 2
Clinical Score 1, Group 2
Clinical Score 0, Group 2

Figure 1

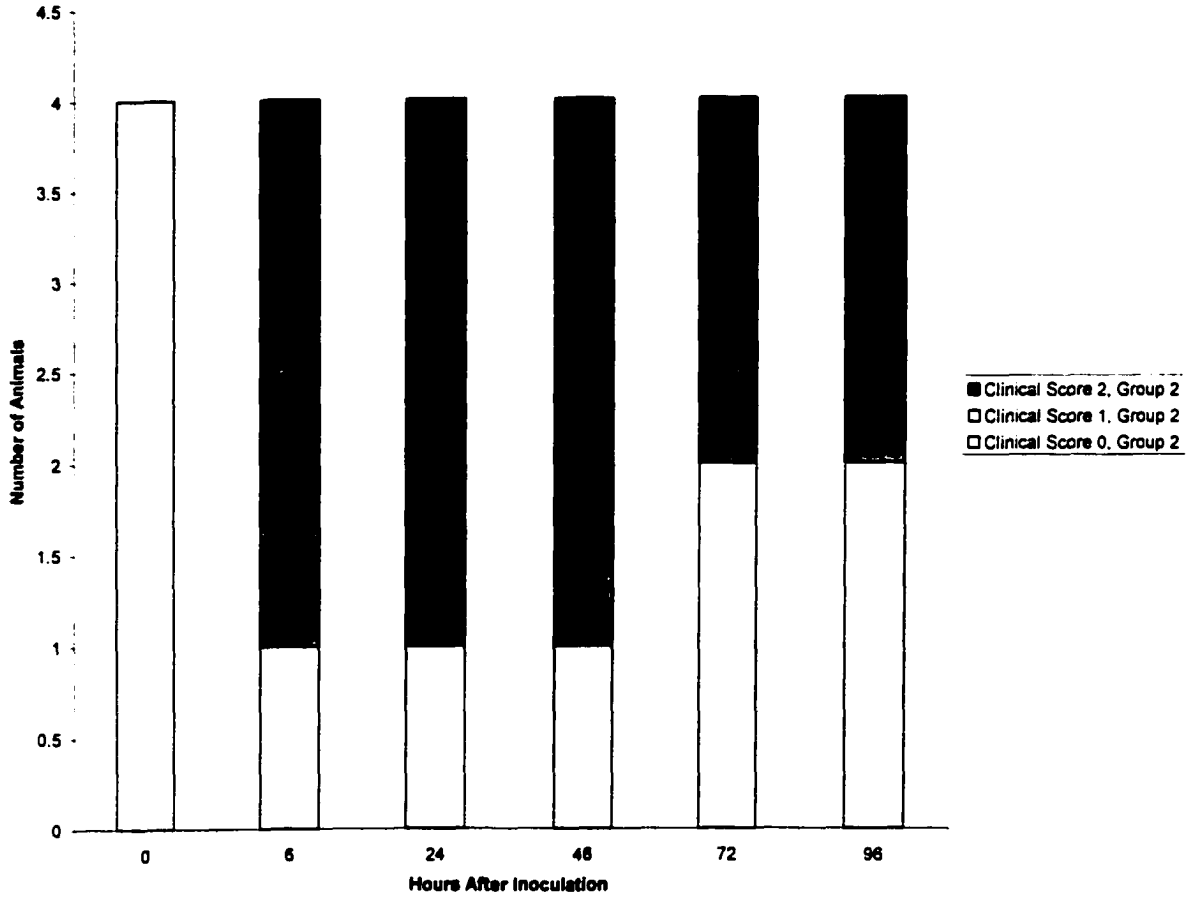
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Number of Animals
0
6
24
48
72
96
Hours After Inoculation
Clinical Score 2, Group 2
Clinical Score 1, Group 2
Clinical Score 0, Group 2

Figure 2

Table 1. Cytology results for bronchoalveolar lavage samples from heifer calves in Groups 2 and 3 as measured at 24 and 48 hours after intrabronchial challenge with 20 cc of bacterial culture fluid containing approximately 1 x10^8 colony forming units/ml of *P. haemolytica.*

	Total leukocyte count (x10^3/μl)	Neutrophil count (x10^3/μl)	Percent neutrophils
Group 2 – 24 hours	0.249	0.157	63.0%
Group 2 – 48 hours	0.288	[a]	17.5%[b]
Group 3 – 24 hours	0.404	0.063	16.0%
Group 3 – 48 hours	0.301	[a]	33.0%[b]

[a]Not counted
[b]Visual estimate

Table 2. Number of beef heifer calves for which lung neutrophil assays could be performed due to lack of neutrophils in the samples. Bronchoalveolar lavages were performed on 8 calves at 24 and 48 hours after intrabronchial challenge with 20 cc of bacterial culture fluid containing approximately 1 x10^8 colony forming units/ml of *P. haemolytica.*

	Neutrophil Assay					
Hours after inoculation	*S. aureus* ingestion	Cytochrome C reduction	Iodination	AICC/ ADCC	Chemotaxis	Random migration
24	3	3	3	3	3	3
48	2	0	2	4	0	2
Total	5/16	3/16	5/16	7/16	3/16	5/16

Table 3. Lung lobe consolidation as estimated visually and by palpation. Mean proportion of each lobe is calculated as is the mean proportion of the entire consolidation from that lobe using the formula described in the text.

Lung lobe	**Mean proportion consolidated of designated lobe ± SD**	**Mean proportion of entire lung consolidation ± SD**
Left Cranial--cranial segment	0.13 ± 0.23	0.028 ± 0.053
Left Cranial--caudal segment	0.33 ± 0.45	0.069 ± 0.092
Left Caudal	0.019 ± 0.037	0.024 ± 0.046
TOTAL LEFT SIDE		0.12 ± 0.19*
ACCESSORY	0.51 ± 0.45	0.13 ± 0.12
Right Caudal	0.19 ± 0.29	0.30 ± 0.35
Right Middle	0.70 ± 0.34	0.37 ± 0.28
Right Cranial	0.063 ± 0.18	0.013 ± 0.038
Right Cranial/cranial segment	0.16 ± 0.27	0.072 ± 0.15
TOTAL RIGHT SIDE		0.75 ± 0.23*

*p=0.0001

CHAPTER 4. THE EFFECTS OF DANOFLOXACIN AND TILMICOSIN ON NEUTROPHIL FUNCTION AND LUNG CONSOLIDATION IN BEEF CALVES WITH INDUCED *PASTEURELLA* PNEUMONIA

A paper prepared for submission to the Journal of Veterinary Pharmacology and Therapeutics

Virginia R. Fajt, Michael D. Apley, James A. Roth, Dagmar E. Frank, Kim A. Brogden, Terry L. Skogerboe, Valerie K. Karle, Arthur D. Dayton

Abstract

Pasteurella pneumonia was induced in weaned beef heifer calves, approximately 6 months of age. Calves were treated at 20 hours after challenge with therapeutic doses of danofloxacin or tilmicosin. Peripheral blood neutrophils were collected at 3, 24 and 48 hours after treatment. The *ex vivo* effects on neutrophil function, neutrophil apoptosis, and hematological parameters were examined, as was the effect on percent 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 assay. Apoptosis was determined using a cell death detection kit. Euthanasia was performed at 72 hours after treatment. Statistical comparisons were made among the 3 groups of challenged-treated animals: saline-, danofloxacin-, and tilmicosin-treated animals. Comparisons were also made between non-challenged non-treated 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 percent lung consolidation among the challenged-treated groups. Significant differences were found between the NCH calves and the challenged non-treated

calves in several neutrophil assays, which were attributed to an effect of the *Pasteurella* infection.

Introduction

The effect of antimicrobials on immune function is a burgeoning area of investigation in veterinary medicine, particularly in the context of diseases such as *Pasteurella* pneumonia in which the inflammatory process appears to have a major effect on clinical outcome. The authors have previously reported on the effects of two antimicrobials, danofloxacin and tilmicosin, on circulating neutrophil function in healthy beef calves. No significant differences were found between the two antimicrobials using assays that evaluated the ability of neutrophils to be recruited and attracted to the site of infection (measured via random migration under agarose and chemotaxis), the ability to phagocytose (measured via *Staphylococcus* ingestion), and the ability to kill bacteria as indicated by superoxide anion production (measured via reduction of Cytochrome C) and the myeloperoxidase- hydrogen peroxide-halide system (measured via an iodination assay) (Fajt et al, submitted). 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 (Hoeben et al., 1997a;Hoeben et al., 1997b;Hoeben et al., 1998;Nickerson et al., 1985;Paape & Miller, 1990;Paape et al., 1991;Ziv et al., 1983).

There has been a report of the in vivo effects of tilmicosin on neutrophils isolated from the lungs of calves with induced pneumonia (Chin et al., 1998). In that study, young beef calves were pre-treated with tilmicosin 18 hours and 15 minutes prior to the induction of *Pasteurella* pneumonia. The authors reported that tilmicosin increased the incidence of

neutrophil apoptosis at 3 hours after infection, but saw no other effects on neutrophil morphology or function. The significance of apoptosis of neutrophils in bovine respiratory disease has not been well established. Much of the damage that occurs to lungs in *Pasteurella* pneumonia in cattle can be attributed to the presence of neutrophils and their associated oxygen radicals and enzymes (Slocombe et al., 1985;Breider et al., 1988;Breider et al., 1986). Therefore, a drug that causes the death of neutrophils through apoptosis might diminish the inflammatory response and damage to lung parenchyma. On the other hand, dead neutrophils cannot perform their microbicidal functions, which might lead to the overwhelming of immune defenses by the infection.

The challenge 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. In vivo studies have shown a decrease in circulating neutrophil chemotaxis associated with *P. haemolytica* infection (Henricks et al., 1987), and an increased chemiluminescence response which is an indication of oxygen-dependent bactericidal activity (Davies et al., 1986). In vitro, leukotoxin from *P. haemolytica* has been shown: to increase the production of leukotriene B_4 (Clinkenbeard et al., 1994;Henricks et al., 1992), to activate the respiratory burst (Ortiz-Carranza & Czuprynski, 1992), to decrease the chemiluminescence response (an indication of oxidative metabolism) (Henricks et al., 1990), and to cause morphologic changes consistent with apoptosis (Stevens & Czuprynski, 1996).

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. Danofloxacin is approved for the treatment

of bovine respiratory disease in Europe and South America, while tilmicosin is approved in the United States and Canada for the same indication. Assessing the function of circulating neutrophils instead of neutrophils that infiltrate the lung has two advantages: the samples are easier to collect, and more importantly, the potential local effect of the *Pasteurella* itself on neutrophil function in situ in the lung is removed as a confounding factor, although systemic effects of *Pasteurella* are not removed. 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 just as they enter the lung. Previous experience in challenged animals indicates that the majority of neutrophils isolated directly from the lung via bronchoalveolar lavage are degenerate and non-viable, and their activity cannot be evaluated (Fajt et al., 1999b). The other objective of this study was to evaluate the two antimicrobials' abilities 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 were pre-conditioned prior to their arrival at the study site 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 that

exhibited any clinical signs of disease before *P. haemolytica* inoculation were removed from eligibility for the study and treated as appropriate.

Animals were housed outdoors in concrete-floored pens with open front sheds as shelter (approximately 11.5 m^2/head). They were fed free choice hay and a grain supplement balanced to allow weight gain of approximately 1.5 lbs/head/day. The grain supplement contained monensin sodium at a rate of approximately 150 mg/head/day. Water was provided ad libitum.

Bacterial Challenge

Bacterial challenge was performed as previously described (Fajt et al., 1999c). A 3-hour log phase culture of a frozen (-70°C) stock culture of *Pasteurella haemolytica* Type A1 strain L101 isolated from a calf with pneumonia was used. The broth culture was adjusted spectrophotometrically to an approximate concentration of 1 x 10^8 colony forming units/ml. Concentrations of bacteria were verified after the inoculation procedure with standard plate counts.

Culture fluid (20 cc) was administered to each calf through an equine bronchoalveolar lavage catheter.[a] The catheter was passed through the nares into the trachea as far as possible into a caudal lung lobe, then withdrawn approximately 20 cm so that inoculation would occur close to the level of the tracheal bifurcation. Culture fluid was placed in the catheter, followed by approximately 180 ml of air pushed in rapidly with a 60 cc syringe.

Selection Criteria

Animals were randomly selected for bacterial challenge from an initial group of 48. Two groups of calves were challenged with *P. haemolytica*: 18 animals during Week 1, and 15 animals during Week 2. Of these animals, 12 were selected each week for treatment,

resulting in a total of 24 challenged-treated animals. At 20 hours after bacterial challenge, animals were clinically scored, and rectal temperatures were measured. The clinical scoring system used has been previously described (Perino & Apley, 1998). Briefly, a score of 0 is a normal animal, 1 is noticeable depression without apparent signs of weakness, 2 is marked depression with moderate signs of weakness without significantly altered gait, 3 is severe depression with signs of weakness such as significantly altered gait, and 4 is moribund and unable to rise.

The successful induction of respiratory disease was defined as a rectal temperature of $\geq 40°C$ and a clinical score of 1 or greater. The protocol dictated that animals that displayed a clinical score of 4 (moribund, unable to rise) were immediately evaluated for euthanasia and were not eligible for the study, but no animals exhibited this score.

Using a randomized complete block design, a random number from a uniform distribution on the interval (0,1) was assigned to each animal meeting the selection criteria after bacterial challenge. Animals meeting entrance criteria were sorted by this random number, and the first 12 were chosen to be used for each week in the study. (Animals not selected were treated appropriately with an antimicrobial.) The selected animals were sorted by temperature and grouped into blocks of 3 from lowest to highest temperature. A random number from a uniform distribution on the interval (0,1) was again assigned to each animal, and the animals were sorted within the 4 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. The above process was repeated for the second challenge group of 15 animals, resulting in a total of 24 challenged and treated animals.

Four animals were also selected each week at random to be non-challenged, non-treated controls (NCH), for a total of 8 NCH calves.

Clinical Scoring

Clinical scoring was performed by an investigator who was not aware of the treatment groups at 0, 24, 48 and 72 hours after treatment. Treatment was at 20 hours post-challenge. The scoring system is described above under selection criteria.

Antimicrobials

Animals received one of the following treatments at 20 hours after bacterial challenge: danofloxacin mesylate[viii] (180 mg/ml) at a dosage of 6 mg/kg subcutaneously in the left lateral neck, tilmicosin[ix] (300 mg/ml) at a dosage of 10 mg/kg subcutaneously in the left lateral neck, and saline (0.9%) in a volume equal to a dose of danofloxacin subcutaneously in the left lateral neck.

Blood Sampling

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

Hematology

Blood samples were collected via jugular venipuncture and placed in tubes containing EDTA[x]. An automated cell counter[xi] 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

[viii] Advocin, Pfizer Inc, New York, New York.
[ix] Micotil, Elanco Animal Health, Indianapolis, Indiana.
[x] Vacutainer, Becton Dickinson and Co., Franklin Lakes, New Jersey.
[xi] Cell-Dyne 3500, Abbott Labs, Abbott Park, Illinois.

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., 1999a). 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 (Fajt et al., 1999a;Lukacs et al., 1985;Roth & Kaeberle, 1981;Roth & Kaeberle, 1981). 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.[xii] Unstimulated levels of apoptosis were determined as was the percent apoptosis after addition of camptothecin (0.1 and 1.0 μg/ml), an apoptosis-inducing agent.

Lung Lesions

Animals were euthanatized with an overdose of barbiturate. At necropsy, lungs were removed from calves, and the percentage of consolidation of each lobe was visually estimated using a formula previously described (Fajt et al., 1999c). Areas of consolidation were sampled for histopathology and bacterial culture.

MIC 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.[xiii] Custom plates containing several dilutions of the following antimicrobials were used: danofloxacin, enrofloxacin, ceftiofur, cefquinome, amoxicillin/clavulanic acid, colistin, spiramycin, oxytetracycline, trimethoprim/sulfadiazine, gentamicin, tilmicosin, spectinomycin, and tylosin.

Statistical Analysis

The following variables were natural log transformed prior to analysis: neutrophil chemotactic index, mean corpuscular volume, lymphocyte absolute count, monocyte absolute count, red cell distribution width, mean platelet volume, and mononuclear white blood cell count. The transformation resulted in residuals that were more normally distributed and also stabilized the variance. The least squares means were then back-transformed after analysis. The repeatedly measured variables from the challenged animals (3 treatment groups) including the neutrophil assay variables, apoptosis assay, and hematology results, were analyzed using a general linear repeated-measures mixed model.[xiv] If a significant difference was detected ($p \leq 0.05$) due to day of study or treatment effect, pairwise comparisons among treatments were made. NCH calves were compared to the saline-treated calves using a t-test. Statistical significance was defined as $p \leq 0.05$. NCH calves could only be compared to saline-treated calves and were not included in the overall general linear model because animals in the challenged groups were blocked by rectal temperature before treatments were assigned, and the non-challenged non-treated animals were not included in those blocks.

[xii] Cell Death Detection ELISA, Roche Molecular Biochemicals, Indianapolis, Indiana.
[xiii] Sensititre, Trek Diagnostic Systems, Westlake, Ohio.
[xiv] SAS Open VMS version 6.12 for an alpha, 1998, SAS Institute Inc., Cary, North Carolina.

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.

Percent 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 if a significant ($p \leq 0.05$) treatment effect was found. After analysis, the least-squares means were back-transformed for presentation.

Descriptive statistics were performed on the MIC values from *P. haemolytica* isolated from the lungs.

Results

Standard plate counts of the *P. haemolytica* challenge strain revealed a concentration of 2.9×10^8 colony forming units/ml in the first group and 1.7×10^8 colony forming units/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. At 24 and 48 hours after treatment, all of the challenged-treated calves had clinical scores of 1 or 2. By 72 hours after treatment, just prior to euthanasia, 2 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 core body temperature (see Chapter 5).

Neutrophil assays

For all of the neutrophil function assays (random migration, cytochrome C, iodination, *Staphylococcus* ingestion, chemotaxis, and cytotoxicity), there was a significant day-of-collection effect. 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 hours after treatment. (See Tables 1-7 for summaries of the neutrophil function data, as well as the power to detect differences for each of the assays and the least significant differences.)

When the NCH calves were compared to 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 at 48 hours after treatment for neutrophil random migration, which was significantly lower in the saline-treated calves than in the NCH calves (50.5 and 90.4 mm^2, respectively). No differences were found at the other time points.

At 24 hours after treatment, neutrophils from the saline-treated calves displayed significantly higher *Staphylococcus* ingestion percentage than neutrophils from NCH calves (29.6 and 20.3%, respectively). No differences were found at the other time points.

At 3 hours after treatment, the percent antibody-independent cytotoxicity was significantly higher for the saline-treated calves than for the NCH calves (20.4 and 2.3%, respectively), but no differences were found at the other time points.

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 (see Table 8).

Hematology

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

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 was significantly lower than saline- or tilmicosin-treated calves at 24 hours after treatment, and than tilmicosin-treated calves at 48 hours after treatment. The difference between saline- and danofloxacin-treated calves also approached significance at 48 hours ($p=0.0522$), with danofloxacin-treated calves being lower. The MCHC for saline-treated calves was significantly lower than that of NCH calves at 24 hours after treatment, but no other differences were found among challenged-treated calves for this parameter.

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 as compared to the NCH group.

Lung Consolidation and Bacteriology

No significant differences for percentage lung consolidation were found among treatment groups or among blocks of animals (blocked by temperature at time of treatment). Total percentage of lung consolidation in individual animals ranged from 2.02 to 29.6%, with means of 18.1, 14.7, and 13.0% for saline-, danofloxacin-, and tilmicosin-treated animals respectively.

P. haemolytica was isolated from 7 of the 8 saline-treated, 8 of the 8 danofloxacin-treated, and 5 of the 8 tilmicosin-treated calves. Growth from 1 tilmicosin-treated calf revealed no *P. haemolytica*, but low numbers of colonies of *Staphylococcus epidermidis* and *P. multocida*. The MICs for the challenge isolate are shown in Table 9. MICs for all of the lung isolates were the same as the challenge inoculum with 2 exceptions: The MIC for spectinomycin was 16 μg/ml for 3 isolates, and the MIC for oxytetracycline was 64 μg/ml for 2 isolates, 1.0 μg/ml for 1 isolate, and ≤ 0.5 μg/ml for 3 isolates.

Histopathological 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* (28/33 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 hours 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 among the same neutrophil function assays between saline-, danofloxacin-, or tilmicosin-treated calves (Fajt et al., 1999a). Since danofloxacin and tilmicosin administered to healthy animals did not affect neutrophil function, then we hypothesized that the addition of induced *Pasteurella* pneumonia to the scheme should not affect neutrophil function differentially between the two antimicrobials either. The finding of no differences among treatment groups in the apoptosis assays is not consistent with the results of Chin et al. (Chin et al., 1998), who found that neutrophils isolated from the lungs of tilmicosin-treated *P. haemolytica*-challenged calves exhibited increased apoptosis at 3 hours after treatment compared to sham-treated animals (although there were no differences at 24 hours after treatment). One major difference between that study and the current one was that the current study used neutrophils isolated from the blood and the former study used neutrophils recovered from the lung. In addition, animals in the former study were treated prior to bacterial challenge and there was not an unchallenged group, making it difficult to determine the role of *P. haemolytica* infection on the induction of apoptosis. It is possible that pre-treatment with antibiotic reduced the growth and metabolism of *P. haemolytica* at 3 hours after challenge, resulting in less *P. haemolytica*-induced apoptosis, rather than a direct effect of tilmicosin on neutrophil apoptosis.

Because no significant differences were found among the challenged-treated groups for the neutrophil assays, we calculated the power to detect these differences. Using a reasonable number of animals (8/treatment group), the power ranged from 0.05-0.65 depending on the assay. This is mostly a result of the variability associated with the

neutrophil function assays. Because power is often not reported in studies similar to this one, there is no basis for comparison. However, due to the expense of the animals and the neutrophil assays, 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, in which differences were found in the random migration assay, the *S. aureus* ingestion assay, and the antibody-independent cytotoxicity assay. Neutrophils from saline-treated calves migrated significantly less than NCH calf neutrophils at the 48-hour time period. Activated neutrophils might be expected to display more directed movement (chemotaxis) rather than random migration under the influence of an infection, although this only appeared to occur close to 72 hours after bacterial challenge. Neutrophils from the saline-treated calves displayed higher *S. aureus* ingestion percentage at the 24-hour time period than NCH calves. Neutrophils from the saline-treated calves also exhibited a higher percent antibody-independent cytotoxicity at the 3-hour time period than NCH calves. The fact that the only differences were between the challenged-saline-treated calves and the NCH calves suggests that these differences are due to the *P. haemolytica* challenge in some manner, perhaps as a result of circulating factors associated with the infection since the neutrophils were collected from the peripheral circulation. Challenged calves had higher core body temperatures as measured via radiotelemetry (see Chapter 5), suggesting pro-inflammatory cytokine release, which could effect changes in neutrophil function. Certainly, *P. haemolytica* and its products have been shown to affect bovine neutrophils in vitro as discussed above.

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 of the laboratory (24-46%). This phenomenon was also found in the authors' previous report on danofloxacin in healthy calves (Fajt et al., 1999a), although it has not been reported elsewhere for danofloxacin. It has been reported for temafloxacin, a human fluoroquinolone, which was removed from the market due to reports of hemolysis with or without organ failure associated with an autoimmunity to erythrocytes (Blum et al., 1994).

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 hours post-inoculation with *P. haemolytica* (Rashid et al., 1997).

The lack of significant differences among the challenged-treated groups in percent 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 to 37.5% consolidation in non-treated animals (Fajt et al., 1999c). Additionally, the time of necropsy (72 hours 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. In a previous experiment evaluating antimicrobial therapy, non-treated animals euthanatized on day 12 after inoculation (day 11 after treatment) had significantly higher percent lung involvement (33±10%) as compared to treated animals (ranged from 4-12%), with no significant differences between different antimicrobials (Ames et al., 1987).

The changes in MICs from challenge inoculum to lung isolate may be due to passage in the lungs; since 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 to conclude that the challenge strain was the same as the isolated strain. Differences of one dilution are considered non-significant, so the isolate exhibiting a MIC to spectinomycin of 16 μg/ml should not be considered different from the challenge strain MIC of 32 μg/ml.

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 *Pasteurella* pneumonia. On the other hand, there is a significant effect of *Pasteurella* infection that results in changes in circulating neutrophil function in the absence of antimicrobial administration.

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Table 1. The distance neutrophils migrated under agarose in mm^2 (neutrophil random migration assay). Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). NCH (non-challenged group) is arithmetic means; all other groups are least squares means. The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Time after treatment	**3 hours**	**24 hours**	**48 hours**
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 ± 10.41	70.1 ± 12.3	90.4 ± 17.9*
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 $p \leq 0.05$

†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 means at $p \leq 0.05$.

Table 2. The distance neutrophils migrated toward a chemotactic substance in mm^2 (neutrophil chemotactic assay) reported as geometric means with 95% confidence intervals, except for NCH which is arithmetic means with standard errors. Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Time after treatment	**3 hours**	**95% CI**	**24 hours**	**95% CI**	**48 hours**	**95% CI**
Saline	1.16	1.05-1.29	1.14	1.03-1.26	1.24	1.12-1.37
Danofloxacin	1.18	1.06-1.30	1.19	1.07-1.31	1.24	1.12-1.38
Tilmicosin	1.11	1.01-1.23	1.13	1.02-1.25	1.29	1.17-1.43
NCH	1.12 ± 0.04		1.12 ± 0.04		1.17 ± 0.06	
Contrast	**LSD† (Power‡)**		**LSD† (Power‡)**		**LSD† (Power‡)**	
Saline vs. danofloxacin	0.196 (0.05)		0.196 (0.09)		0.196 (0.05)	
Saline vs. tilmicosin	0.196 (0.10)		0.196 (0.05)		0.196 (0.10)	
Danofloxacin vs. tilmicosin	0.196 (0.13)		0.196 (0.11)		0.196 (0.09)	

†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 means at $p \leq 0.05$.

Table 3. Percent radiolabeled *S. aureus* ingested by neutrophils (*Staphylococcus* ingestion assay). Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). NCH is arithmetic means; all other groups are least squares means with standard errors. The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Time after treatment	**3 hours**	**24 hours**	**48 hours**
Saline	34.4 ± 2.3	29.6 ± 2.4*	18.0 ± 4.2
Danofloxacin	35.3 ± 2.3	30.9 ± 2.4	14.0 ± 4.2
Tilmicosin	34.0 ± 2.3	30.1 ± 2.4	18.4 ± 4.2
NCH	29.9 ± 2.6	20.3 ± 4.6*	7.1 ± 2.0
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)

*Differences significant at p≤0.05
†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 means at p≤0.05.

Table 4. Percent iodination as measured using opsonized zymosan (iodination assay); evaluates myeloperoxidase/hydrogen peroxide/halide system of neutrophils. Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). NCH is arithmetic means; all other groups are least squares means with standard errors. The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Time after treatment	**3 hours**	**24 hours**	**48 hours**
Saline	15.1 ± 3.2	16.2 ± 2.4	15.5 ± 3.2
Danofloxacin	18 ± 3.2	15.1 ± 2.4	14.3 ± 3.2
Tilmicosin	12.9 ± 3.2	16.1 ± 2.4	17.3 ± 3.2
NCH	21.3 ± 2.9	17.6 ± 2.2	20.0 ± 2.9
Contrast	**LSD† (Power‡)**	**LSD† (Power‡)**	**LSD† (Power‡)**
Saline vs. danofloxacin	11.7 (0.11)	7.7 (0.07)	11.7 (0.06)
Saline vs. tilmicosin	11.7 (0.09)	7.7 (0.05)	11.7 (0.07)
Danofloxacin vs. tilmicosin	11.7 (0.24)	7.7 (0.07)	11.7 (0.11)

†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 means at $p \leq 0.05$.

Table 5. Optical density of neutrophils incubated with Cytochrome C and opsonized zymosan (Cytochrome C reduction assay). Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). NCH is arithmetic means; all other groups are least squares means with standard errors. The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Time after treatment	**3 hours**	**24 hours**	**48 hours**
Saline	0.51 ± 0.04	0.36 ± 0.04	0.41 ± 0.04
Danofloxacin	0.49 ± 0.04	0.33 ± 0.04	0.40 ± 0.04
Tilmicosin	0.46 ± 0.04	0.37 ± 0.04	0.45 ± 0.04
NCH	0.44 ± 0.06	0.28 ± 0.06	0.35 ± 0.06
Contrast	**LSD† (Power‡)**	**LSD† (Power‡)**	**LSD† (Power‡)**
Saline vs. danofloxacin	0.16 (0.07)	0.16 (0.07)	0.16 (0.06)
Saline vs. tilmicosin	0.16 (0.15)	0.16 (0.06)	0.16 (0.10)
Danofloxacin vs. tilmicosin	0.16 (0.08)	0.16 (0.11)	0.16 (0.14)

†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 means at $p \leq 0.05$.

Table 6. Percent lysis of radiolabeled chicken erythrocytes (cRBC) in the presence of anti-cRBC antibody. Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). NCH is arithmetic means; all other groups are least squares means with standard errors. The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Time after treatment	**3 hours**	**24 hours**	**48 hours**
Saline	40.8 ± 3.8	30.9 ± 3.8	38.7 ± 3.8
Danofloxacin	41.6 ± 3.8	32.4 ± 3.8	34.1 ± 3.8
Tilmicosin	43.1 ± 3.8	25.6 ± 3.8	35.0 ± 3.8
NCH	38.1 ± 3.5	27.1 ± 3.3	30.2 ± 6.0
Contrast	**LSD† (Power‡)**	**LSD† (Power‡)**	**LSD† (Power‡)**
Saline vs. danofloxacin	15.8 (0.05)	15.8 (0.06)	15.8 (0.13)
Saline vs. tilmicosin	15.8 (0.07)	15.8 (0.16)	15.8 (0.10)
Danofloxacin vs. tilmicosin	15.8 (0.06)	15.8 (0.23)	15.8 (0.05)

†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 means at $p \leq 0.05$.

Table 7. Percent lysis of radiolabeled chicken erythrocytes (cRBC) without anti-cRBC antibody. Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). NCH is arithmetic means; all other groups are least squares means with standard errors. The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Time after treatment	**3 hours**	**24 hours**	**48 hours**
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	11.0 ± 4.2	10.8 ± 3.9
NCH	2.3 ± 1.4*	1.4 ± 0.7	0.7 ± 0.5
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)

*Differences significant at p≤0.05

†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 means at p≤0.05.

Table 8. Cell death as detected using a commercially-available cell death detection kit. Unstimulated levels of apoptosis were determined, as was the percent apoptosis after addition of camptothecin (0.1 and 1.0 μg/ml), an apoptosis-inducing agent. Neutrophils were collected at 3, 24 and 48 hours after treatment with danofloxacin or tilmicosin (approximately 23, 44 and 68 hours after challenge with *P. haemolytica*). NCH is arithmetic means; all other groups are least squares means with standard errors. The lower portion of the table shows the least significant difference required between the groups to show a statistical difference, along with the statistical power to detect a difference.

Camptothecin concentration	**0 ug/ml**			**0.1 ug/ml**			**1.0 ug/ml**		
Time after treatment	3 hours	24 hours	48 hours	3 hours	24 hours	48 hours	3 hours	24 hours	48 hours
Saline	0.32 ± 0.06	0.36 ± 0.06	0.34 ± 0.06	0.65 ± 0.18	0.79 ± 0.18	0.99 ± 0.18	1.15 ± 0.24	1.34 ± 0.16	2.23 ± 0.40
Danofloxacin	0.3 ± 0.06	0.4 ± 0.06	0.4 ± 0.06	1.03 ± 0.18	1.2 ± 0.18	1.09 ± 0.18	1.24 ± 0.24	1.49 ± 0.16	1.68 ± 0.40
Tilmicosin	0.43 ± 0.06	0.47 ± 0.06	0.47 ± 0.06	0.82 ± 0.18	0.92 ± 0.18	1.0 ± 0.18	1.04 ± 0.24	1.37 ± 0.16	1.98 ± 0.40
NCH	0.42 ± 0.07	0.54 ± 0.04	0.44 ± 0.05	0.96 ± 0.18	0.82 ± 0.13	0.81 ± 0.11	1.39 ± 0.30	1.13 ± 0.13	1.28 ± 0.14
LSD† (Power‡)									
Saline vs. danofloxacin	0.25 (0.05)	0.25 (0.08)	0.25 (0.11)	0.67 (0.37)	0.66 (0.43)	0.66 (0.07)	0.95 (0.06)	0.62 (0.10)	1.61 (0.17)
Saline vs. tilmicosin	0.25 (0.25)	0.25 (0.25)	0.25 (0.30)	0.69 (0.11)	0.66 (0.09)	0.66 (0.05)	0.95 (0.06)	0.62 (0.05)	1.61 (0.07)
Danofloxacin vs. tilmicosin	0.25 (0.31)	0.25 (0.13)	0.25 (0.11)	0.69 (0.14)	0.66 (0.23)	0.66 (0.07)	0.95 (0.09)	0.62 (0.08)	1.61 (0.08)

†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 means at $p \leq 0.05$.

Table 9. Minimum inhibitory concentrations in micrograms/ml of the challenge inoculum of *P. haemolytica* to antimicrobials, as evaluated using the Sensititre® susceptibility testing system.

Antimicrobial	Minimum Inhibitory Concentration (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

CHAPTER 5. THE EFFECT OF DANOFLOXACIN AND TILMICOSIN ON CORE BODY TEMPERATURES IN BEEF CALVES WITH INDUCED *PASTEURELLA* PNEUMONIA

A paper prepared for submission to the American Journal of Veterinary Research

Virginia R. Fajt, Michael D. Apley, Kim A. Brogden,
Terry L. Skogerboe, Valerie K. Karle, Arthur D. Dayton

Abstract

Objective

To examine the effect of treatment of induced *P. haemolytica* pneumonia in beef calves with danofloxacin and tilmicosin on continuously recorded body temperature.

Animals

32 weaned Angus-cross beef heifers (160-220 kg) from a single ranch with no recent history of respiratory disease or antimicrobial therapy.

Procedure

Pneumonia was induced intrabronchially using log-phase cultures of *Pasteurella haemolytica*. At 20 hours post-challenge, animals were treated with saline, danofloxacin or tilmicosin. Radiotransmitters were implanted intravaginally, and core body temperatures were monitored from 66 hours prior to challenge until necropsy at 72 hours after treatment. For each animal, a baseline temperature was established based on pre-challenge body temperature. The area under the curve of the temperature-time plot was calculated for 3-hour intervals, and compared across treatment groups. Mean 3-hour interval temperatures were also calculated.

Results

Areas under the curve for 3-hour intervals were not significantly different for any of the time intervals when challenged-treated animals were compared. Analysis of the mean 3-hour interval temperatures showed significantly higher temperatures for saline-treated as compared to antimicrobial-treated animals for the majority of the time intervals, but no differences were found between the danofloxacin- or tilmicosin-treated animals. The circadian rhythm of temperatures pre-challenge returned at approximately 48 hours after challenge.

Conclusions

Danofloxacin and tilmicosin did not differ in their effect on mean 3-hour interval core body temperatures, but significantly decreased temperatures as compared to controls.

Clinical Relevance

Normal daily variation in core body temperature must be considered in the face of respiratory disease when clinical evaluation of feedlot animals is required.

Introduction

Body temperature as measured by rectal thermometer is a commonly used parameter for evaluating the health status of cattle. Finding an elevated temperature in the presence of other signs of disease is often categorized by the clinician as "fever." A true fever has been defined as "a pyrogen-mediated rise in body temperature above the normal range,[1]" implying a complex physiologic response to, for example, invading bacterial organisms in the case of bovine respiratory disease caused by *Pasteurella haemolytica*. Typically, fever has been measured at a single or a few time points in cattle under

experimental conditions, because taking an animal's temperature can be physically difficult and time-consuming.

Continuous monitoring techniques utilizing radiotelemetry in cattle have been available for over 30 years and have allowed the gathering of temperature data in the absence of restraint. Most reports have involved the surveillance of normal body temperatures such as change in temperature during ovulation or under tropical or summer conditions. Radiotransmitters have been implanted under the skin, in the peritoneum, within the udder, and vaginally in order to monitor body temperature.[2-5] The use of radiotelemetry to monitor temperature in diseased cattle has been reported in induced mastitis models in dairy cows [5,6] and in respiratory viral challenge studies.[4,7] Use of continuous temperature monitoring in disease models has the potential to allow comparison of pharmacotherapeutics or immunologic interventions (Fajt et al., submitted).

The objective of this study was to examine the effect of danofloxacin or tilmicosin treatment of induced *P. haemolytica* pneumonia in beef calves on continuously recorded body temperature.

Materials and Methods

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

Animals

Animals were approximately 6 month old Angus-cross heifers weighing an average of 181.3 kg (SD=19.0) on arrival, purchased as a group from a single herd in Nebraska. Calves were pre-conditioned prior to their arrival at the study site 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 that exhibited any clinical signs of disease before *P. haemolytica* inoculation were removed from eligibility for the study and treated as appropriate.

Bacterial Challenge

Bacterial challenge was performed using the method of Fajt et al. (submitted). Briefly, a 3-hour log phase culture of a frozen (-70°C) stock culture of *Pasteurella haemolytica* A1 strain L101 isolated from a calf with pneumonia was used. The broth culture was adjusted spectrophotometrically to an approximate concentration of 1 x 10^8 colony forming units/ml. Concentrations of bacteria were verified after the inoculation procedure with standard plate counts. Culture fluid (20 cc) was administered intrabronchially to each calf through an equine bronchoalveolar lavage catheter[a] as previously described (Fajt et al., submitted).

Selection Criteria

Animals were selected at random for bacterial challenge as previously described (Fajt et al., submitted). Two groups of calves were challenged with *P. haemolytica*: 18 animals during Week 1, and 15 animals during Week 2, from which 12 animals were selected each week.

At 20 hours after bacterial challenge, animals were clinically scored, and rectal temperatures were measured. The clinical scoring system used has been published.[8] Briefly, a score of 0 is a normal animal, 1 is noticeable depression without apparent signs of weakness, 2 is marked depression with moderate signs of weakness without significantly altered gait, 3 is severe depression with signs of weakness such as significantly altered gait, and 4 is moribund and unable to rise.

The successful induction of respiratory disease was defined as a rectal temperature of ≥ 40°C and a clinical score of 1 or greater, meaning noticeable to severe depression with or without signs of weakness. The protocol stated that animals that displayed a clinical score of 4 (moribund, unable to rise) would be immediately evaluated for euthanasia and were not eligible for the study, although no animals in this study exhibited a score of 4. The study was a randomized complete block design, and animals were assigned to treatment as previously described (Fajt, et al., submitted).

Four animals were also selected at random to be non-challenged, non-treated controls, for a total of 8 NCH calves.

Antimicrobials

Animals received one of the following treatments at 20 hours after bacterial challenge: danofloxacin mesylate[xv] (180 mg/ml) at a dosage of 6 mg/kg subcutaneously in the left lateral neck, tilmicosin[xvi] (300 mg/ml) at a dosage of 10 mg/kg subcutaneously in the left lateral neck, and saline (0.9%) in a volume equal to a dose of danofloxacin subcutaneously in the left lateral neck.

Radiotelemetry

Temperatures were monitored using VHF transmitters[xvii] encased in paraffin using a system described in Fajt et al. (model development study, submitted). The transmitters were implanted at least 3 days prior to inoculation of *P. haemolytica* as follows: A low caudal epidural was performed using 3 cc of lidocaine,[xviii] and then the transmitter was lubricated

[xv] Advocin, Pfizer Inc, New York, New York.
[xvi] Micotil, Elanco Animal Health, Indianapolis, Indiana.
[xvii] Minimitter® Company, Sunriver, Oregon
[xviii] Lidocaine 2% Injectable, Abbott Laboratories, North Chicago, Illinois

with sterile jelly[xix] and placed intravaginally cranial to the vestibular muscle to ensure retention. The software was programmed to read body temperature every 5 minutes.

Statistical Analysis

The radiotransmitters were re-calibrated after study completion by the supplier, and correction factors were calculated for each transmitter to account for drift in temperature over the battery life of the transmitters. Because the transmitters are subject to electromagnetic interference, a procedure was established to remove outliers that did not fit with the surrounding data and that were therefore considered anomalous. First, observations of ≤ 37.5°C or ≥ 42.5°C were considered outside of the normal physiological range and were removed from the data set. Then, an overall mean and standard deviation of the temperatures over the entire period were calculated. If an observation was greater than 3 standard deviations from the overall mean, the value was removed from the data set. Analysis was then performed on the subset of calibrated data with outliers removed.

Two methods of analysis were used to compare core body temperatures among the treatment groups: area under the curve for 3-hour intervals and mean 3-hour interval temperatures. For area under the curve calculations, an average core body temperature (in °C) over at least 48 hours was calculated for each animal from the pre-challenge temperatures. This average minus 1°C was used as a baseline for that animal. The area under the curve (AUC) above the baseline for each animal was calculated for each 3-hour interval pre- and post-challenge, and geometric means of the areas under the curve were calculated. For the mean 3-hour interval temperatures, the arithmetic mean temperature was also calculated for each animal within each 3-hour interval. Analysis of the 3-hour AUC and

[xix] K-Y® Lubricating Jelly, Johnson&Johnson Medical Inc., Arlington, Texas

the mean 3-hour interval temperatures for the 3 challenged-treated groups were analyzed using a general linear repeated-measures mixed model. These variables were also compared with a t-test to test for significant differences between the saline-treated and the NCH calves. NCH calves could only be compared to saline-treated calves and were not included in the overall general linear model because animals in the challenged groups were blocked by rectal temperature before treatments were assigned, and the non-challenged non-treated animals were not included in those blocks.

Results

The temperature profile for a single calf over the entire study is exemplified in Figure 1. This profile is typical, with a large peak in temperature within an hour after challenge, followed by gradually decreasing temperatures post-challenge and once treatment begins. An average of 96% (range 99.94-84.58%) of the data points collected for each animal were usable after the elimination of outliers.

Rectal temperature data were available at the time that animals were selected for inclusion and were compared to transmitter temperature at the same time period. The average difference between transmitter and rectal temperature was 0.36°C, with a range of 0.02-0.80°C. The transmitter temperature was lower than the rectal temperature for only 2 calves.

3-hour AUC Analysis

The time periods of the trial were divided into pre-challenge, post-challenge (pre-treatment) and post-treatment. Analysis of AUC at 3-hour intervals revealed no significant differences among the saline-, danofloxacin-, or tilmicosin-treated groups at any time interval pre- or post-challenge. The mean 3-hour interval AUCs for each treatment group are shown

in Figure 2. Comparing the AUCs for the saline-treated vs. NCH calves revealed significantly lower temperatures in the NCH calves from the first 3-hour interval after challenge until the 21-24 hour interval post-treatment, and at the 27-30 hour interval post-treatment. After 30 hours post-treatment, 3-hour interval AUCs of saline-treated and NCH groups were not significantly different.

Mean 3-hour Interval Analysis

Analysis of the mean 3-hour interval temperatures revealed significant differences at many time intervals between the saline-treated and either of the antimicrobial-treated groups (Figures 3 and 4). The pre-challenge temperatures at the 66-63, 63-60, and 57-54 hour intervals were significantly lower for the tilmicosin-treated group compared to the saline-treated group: 39.2, 39.1, and 39.1°C, and 39.7, 39.5, and 39.4°C for the three intervals for tilmicosin- and saline-treated groups respectively. No other differences were noted among treatment groups pre-challenge.

Post-challenge but before treatment, danofloxacin-treated calves had lower mean 3-hour interval temperatures compared to saline-treated calves at 9-12 hours post-challenge, and tilmicosin-treated calves had lower mean 3-hour interval temperatures compared to saline-treated calves from the 3-6 hour until the 18-21 hour intervals (Figure 4). Danofloxacin- and tilmicosin-treated groups differed at the 12-15, 15-18, and 18-21 hour intervals post-challenge, with the tilmicosin-treated calves having significantly lower mean interval temperatures than the danofloxacin-treated calves. NCH calves had significantly lower temperatures than saline-treated calves from the 3-6 hour until the 18-21 hour interval post-challenge.

Danofloxacin-treated calves had lower mean 3-hour interval temperatures than saline-treated calves from the 3-6 until the 30-33 hour interval post-treatment, from the 36-39 until the 48-51 hour interval, and for the 54-57 hour and the 69-72 hour intervals post-treatment (Figure 4). Tilmicosin-treated calves had lower mean 3-hour interval temperatures than saline-treated calves from the 3-6 until the 45-48 hour interval post-treatment, and for the 60-63 hour until the 69-72 hour interval post-treatment. At no time interval post-treatment were there significant differences between the danofloxacin-treated and the tilmicosin-treated groups.

The mean 3-hour interval temperatures for the saline-treated calves were significantly higher than NCH calves starting from the beginning interval until the 45-48 hour interval post-treatment. The mean interval temperatures of saline-treated calves were also higher from 63-72 hours post-treatment.

Discussion

The temperature time course of the untreated calves in this study followed an expected pattern: Pre-challenge, the calves experienced circadian rhythms as previously described with continuous radiotelemetry[2,9]. In one study[9], body temperature was measured over the winter, and daily minimal temperatures occurred at approximately 8am and daily maximal temperatures at approximately 7pm.

3-hour AUC Analysis

One purpose of this study was to evaluate 2 methods of comparing body temperature data. 3-hour AUC calculations were used to attempt to remove individual variability in normal body temperature by calculating a baseline for each animal pre-challenge. Comparisons of areas under the curve of 3-hour intervals revealed no differences

among the challenged-treated groups of calves for any of the time periods pre- or post-treatment. It did reveal significant differences between the saline-treated and the NCH calves from challenge until 24 hours post-treatment. This is expected since there should be large differences in core body temperature between non-challenged and challenged animals, but it appears that AUC as calculated here may not be sensitive enough to evaluate smaller differences in core body temperatures.

Mean 3-hour Interval Analysis

In the current study, the lowest mean 3-hour interval temperatures occurred during the 6am-9am time interval in the 3 days prior to *P. haemolytica* challenge, and the highest temperatures occurred during the 3pm-6pm interval (Figure 3). An interesting exception to the time of lowest temperatures was in the NCH calves on the day of treatment: the mean interval temperature at the 6am-9am time interval was similar to the immediately preceding and succeeding intervals. This is likely due to the fact that all animals were moved from the pens to the working facility 100 yards away at approximately 5am to be treated, with the temperature increase in the NCH calves associated with increased activity. The circadian rhythm appeared to return to the challenged animals at 48 hours after challenge, when minimal temperatures occurred at the 9am-12pm interval and maximal temperatures at the 3pm-6pm or 6pm-9pm intervals. To the authors' knowledge, this is the first description of interval body temperatures in cattle for any significant period of time after bacterial infection, so it is not known when the return to circadian rhythm is expected.

It is not clear why the tilmicosin-treated group had some significantly lower mean 3-hour interval temperatures than the danofloxacin-treated and saline-treated groups post-

challenge but before treatment. Animals were handled in the same manner and received the same challenge dose of *P. haemolytica*, so an explanation is not apparent.

Significant differences in mean 3-hour interval temperatures are shown in Figure 4. Daily rectal temperatures were significantly different in treated as compared to non-treated animals in one study of induced *P. haemolytica* pneumonia,[10] but were not significantly different in another.[11] One possible explanation for a lack of differences between treated and untreated animals is a difference in disease severity at the onset of treatment; for example, an increase in bacterial challenge dose has been correlated with an increased duration of elevated rectal temperatures.[12] However, in this study, treated animals did have significantly lower mean 3-hour interval temperatures at the majority of the time intervals.

It is possible that the group averages of mean interval temperatures eliminated any individual animal's normal temperature differences and that it was therefore unnecessary to perform the AUC calculations to attempt to eliminate that variation. However, as noted above, the tilmicosin-treated group had lower temperatures than the other groups at several intervals pre-challenge as well as post-challenge but pre-treatment. This implies individual variability in daily temperatures that was not eliminated by using means for the group. Another method of evaluating temperature and other cyclical clinical data that could be applied to these data is time series analysis. This method of analysis has been proposed for use in critical care monitoring in human hospitals as a way of identifying outlying data that require intervention[13] and was utilized in the previously mentioned study on body temperature in feedlot steers.[9] Our intent in this study, however, was to utilize a relatively simple method of analysis without relying on advanced mathematical techniques.

In conclusion, danofloxacin and tilmicosin did not differ in their effects on body temperature in this model of induced *Pasteurella* pneumonia. There were no significant differences among the challenged groups when 3-hour AUC was used as a variable, whereas differences were noted when mean 3-hour interval temperatures were compared. There were, however, significant differences found between the non-treated non-challenged (NCH) calves when compared to the saline-treated calves for both AUC and mean 3-hour interval temperatures. Further work with other disease models might illuminate whether AUC or mean interval temperatures are more appropriate variables to use in the comparison of treatments.

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Figure 1

Example of time-temperature profile over the entire trial as measured via intravaginal radiotransmitter after outliers were removed (see text for explanation). This animal was treated with danofloxacin. The vertical lines in the figure represent the time of challenge and the time of treatment.

Figure 2

Area under the time-temperature curve for 3-hour intervals for saline-, danofloxacin-, and tilmicosin-treated calves and NCH calves; *P. haemolytica* challenge was administered at 0 hours. The hour on the graph corresponds to the beginning hour of the 3-hour interval over

which area under the curve was determined. Standard error bars were not included in the figure, but the range was 1.12-1.14.

Figure 3

3-hour interval mean temperatures for saline-, danofloxacin-, and tilmicosin-treated calves, and NCH calves; *P. haemolytica* challenge was administered at 0 hours. The hour on the graph corresponds to the beginning hour of the 3-hour interval over which temperatures were averaged. Standard error bars were not included in the figure, but the range was 0.138-0.146.

Figure 4

Statistically significant differences in 3-hour interval mean temperatures for saline-, danofloxacin-, and tilmicosin-treated calves, and NCH calves. Radiotransmitters were inserted at time interval -66, *P. haemolytica* challenge was administered at 0, and treatment was administered at 21 hours. The hour on the graph corresponds to the beginning hour of the 3-hour interval over which temperatures were averaged. See Figure 3 for actual temperature differences among groups.


42.5
42
41.5
41
40.5
40
39.5
39
38.5
Temperature (degrees C)
0
200
400
600
800
1000
1200
1400
1600
1800
Observation Number (approx. every 5 minutes)

Figure 1

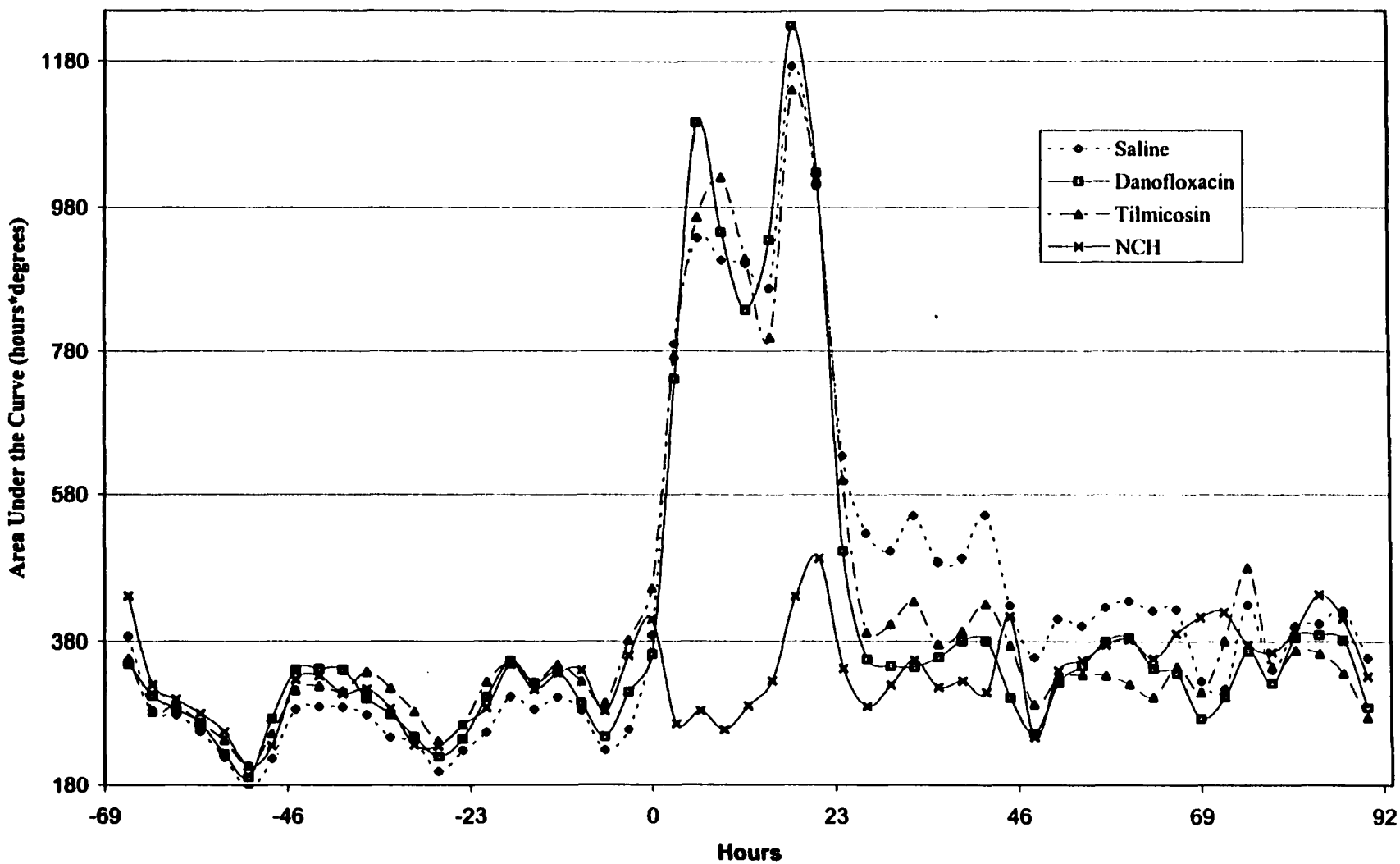

Area Under the Curve (hours*degrees)
1180
980
780
580
380
180
-69
-46
-23
0
23
46
69
92
Hours
Saline
Danofloxacin
Tilmicosin
NCH

Figure 2

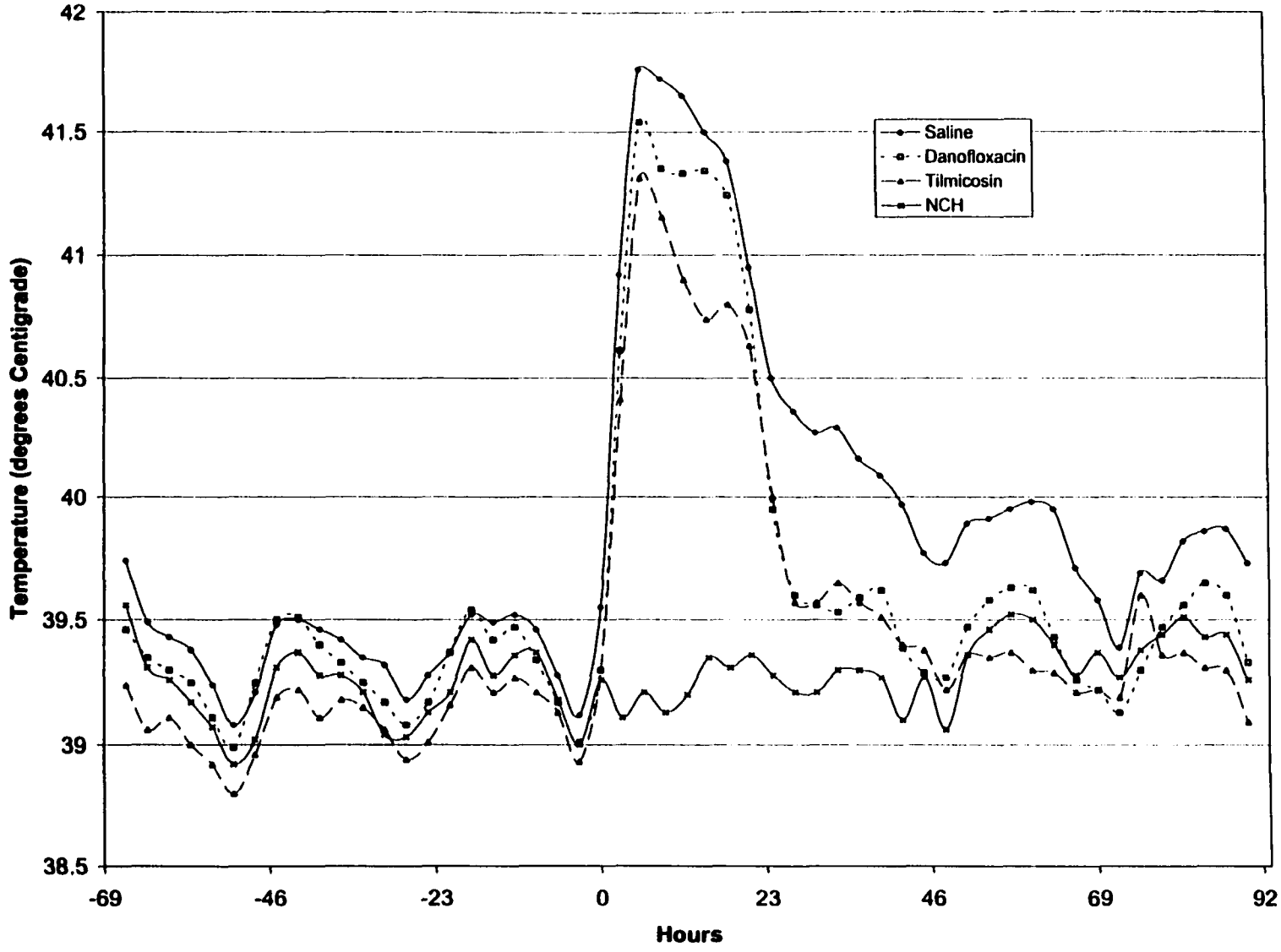

42
41.5
41
40.5
40
39.5
39
38.5
Temperature (degrees Centigrade)
-69
-46
-23
0
23
46
69
92
Hours
Saline
Danofloxacin
Tilmicosin
NCH

Figure 3

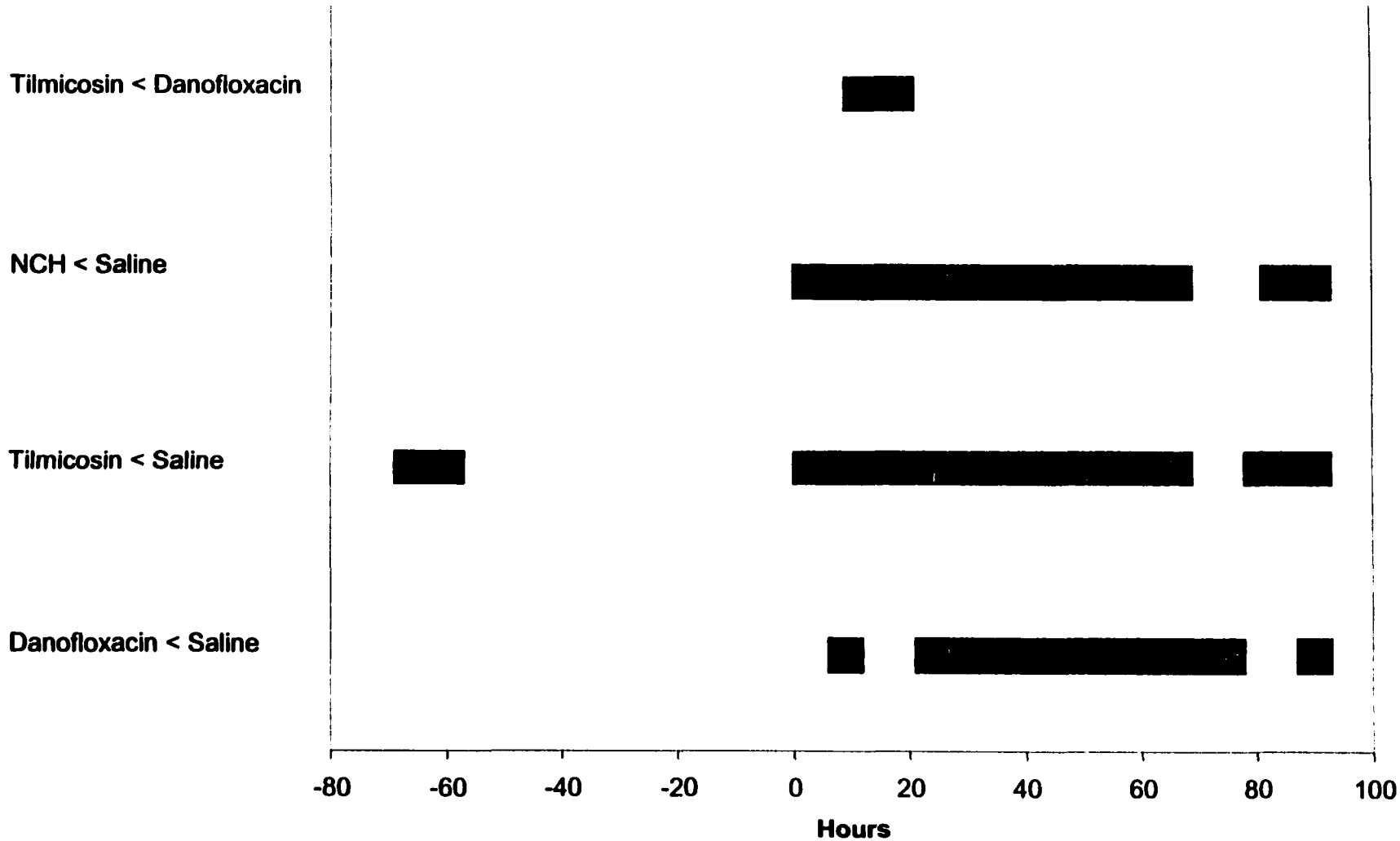

Tilmicosin < Danofloxacin
NCH < Saline
Tilmicosin < Saline
Danofloxacin < Saline
-80
-60
-40
-20
0
20
40
60
80
100
Hours

Figure 4

CHAPTER 6. GENERAL CONCLUSIONS

The overall goals of the research presented in this dissertation pertain to the investigation of the effects of antimicrobials on immune function in cattle. In this case, we examined the effects of danofloxacin and tilmicosin on neutrophil function and apoptosis in healthy and diseased cattle. In addition to examining neutrophil function, the effects of *Pasteurella* pneumonia and treatment with danofloxacin and tilmicosin on core body temperature as measured via radiotelemetry were examined.

In the first study, the effects of danofloxacin and tilmicosin on circulating neutrophil function were examined in clinically normal animals. The results from this study suggest that at therapeutic drug concentrations, danofloxacin and tilmicosin have relatively little effect on the functional abilities of circulating neutrophils in healthy animals.

In the second study, we successfully developed a method of inducing *Pasteurella* pneumonia easily and consistently in weaned beef heifers by instilling live log-phase *Pasteurella haemolytica* cultures intrabronchially using a bronchoalveolar lavage catheter. Using this method, inoculation with *P. haemolytica* resulted in greater than 80% of calves developing clinical scores of ≥ 1, rectal temperatures of $\geq 40°C$, and measurable lung consolidation from which *Pasteurella haemolytica* could be cultured. It was also determined that virtually continuous measurement of core body temperature via radiotelemetry was possible in heifer calves without surgical placement of transmitters. It was also determined that lung neutrophils collected via bronchoalveolar lavage were not sufficiently viable to allow evaluation of neutrophil function.

In the third study, the effects of danofloxacin and tilmicosin on circulating neutrophil function were examined in animals with induced *Pasteurella* pneumonia. The

results suggest that there are no major, clinically significant effects of either danofloxacin or tilmicosin on circulating neutrophil function and apoptosis in this model of induced *Pasteurella* pneumonia. However, it was determined based on comparisons between non-challenged animals and challenged animals that received no antimicrobial that there is a significant effect of *Pasteurella* infection that results in changes in circulating neutrophil function.

In the fourth study, the effects of danofloxacin and tilmicosin on core body temperature in induced *Pasteurella* pneumonia measured via radiotelemetry were examined. The two antimicrobials did not differ in their effects on body temperature in this model of pneumonia, although there were significant differences between the antimicrobial-treated animals and the saline-treated animals when comparing mean 3-hour interval temperatures. No differences were noted between antimicrobial-treated and saline-treated animals when 3-hour AUC was used as a variable. As expected, significant differences were found between the non-treated non-challenged calves when compared to the saline-treated calves for both AUC and mean 3-hour interval temperatures, since non-challenged animals were not subject to the bacterial infection.

The results from these experiments suggest further research in the following areas: In the area of neutrophil function and interaction with pharmaceuticals, work is needed in the investigation of other antimicrobials and their effects on neutrophil function in healthy and diseased animals, the investigation of the effects of danofloxacin and tilmicosin on neutrophils in other disease models in cattle, validation of the no effects findings using larger numbers of animals since our power to detect differences was low in some assays, further investigation of the effects of *Pasteurella* infection on neutrophil function and apoptosis in

the absence and presence of antimicrobials, and evaluation of the long-term effects on neutrophil function as pneumonia and clinical signs resolve (since our studies only examined neutrophils until 48 hours after treatment). In the area of body temperature measurement, further work with other disease models might illuminate whether AUC or mean interval temperatures are more appropriate variables to use in the comparison of treatments. In addition, radiotelemetry in feedlot animals might be used to evaluate vaccine effects on body temperature and subsequent immune response, to follow animals further along in the course of pneumonia resolution to correlate temperature response to feed intake or lesions at slaughter, and to determine at what point animals begin to show clinical signs of pneumonia in relation to their core body temperature.

APPENDIX 1. INSTRUCTIONS FROM CELL DEATH DETECTION KIT.

Cell Death Detection ELISA

Photometric enzyme immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death

Cat. No. 1 544 675
For 96 tests

Version 3, July 1999
Store at 2-8°C

Contents

1. Introduction

Two distinct forms of eukaryotic cell death can be classified by morphological and biochemical criteria: necrosis and apoptosis (1, 2). Necrosis is accompanied by increased ion permeability of the plasma membrane; the cells swell and the plasma membrane ruptures within minutes (osmotic lysis). Apoptosis is characterized by membrane blebbing (zeiosis), condensation of cytoplasma and the activation of an endogeneous endonuclease. This Ca^{2+} and Mg^{2+} dependent nuclease cleaves double stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. In contrast, the DNA of the nucleosomes is tightly complexed with the core histones H2A, H2B, H3 und H4 and is therefore protected from cleavage by the endonuclease (3, 4). The DNA fragments yielded are discrete multiples of an 180 bp subunit which is detected as a "DNA ladder" on agarose gels after extraction and separation of the fragmentsted DNA. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cell is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown (5).

Apoptosis is the most common form of eukaryotic cell death. It occurs e.g. during embryogenesis in parallel with the deletion of autoreactive T cells during thymic maturation, in senescence of neutrophil polymorphs, and following removal of specific growth factors, like IL-2, or the addition of physiological stimmuli like tumor necrosis factor and glucocorticoids (6,7). Apoptosis is also induced by cytotoxic T lymphocytes and natural killer (NK) cells (8,9) and by ionizing radiation (10) and monoclonal antibodies like anti-Fas (11) und anti-APO-1 (12,13).

Advantages of the Cell Death Detection ELISA:

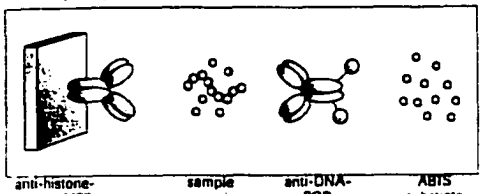
- No radioisotopes are used.
- Quantitative measurement of cell death.
- No prelabeling of the cells required; this allows the quantification of apoptosis also in cells which do not proliferate *in vitro*, e.g. freshly isolated, *ex vitro* tumor cells and organ explants.
- Detection of histone-associated DNA fragments in one immunoassay, demostrating the internucleosomal degradation of genomic DNA occuring during apoptosis.
- The antibodies used are not species specific: Determination of apoptosis in cell systems from various species.
- Results obtained correlate to those obtained by standard methods.
- Highly sensitive; less cells required to obtain results.

2. Product description

2.1 Kit contents

Bottle	Cap	Label	Content
1	white	Anti-histone	Monoclonal antibody from mouse (clone H11-4); lyophilized; stabilized.
2	red	Anti-DNA-POD	Monoclonal antibody from mouse (clone MCA-33), conjugated with peroxidase; lyophilized; stabilized.
3	white	Coating buffer	10 × conc.; solution, 2 ml.
4	green	Washing buffer	10 × conc.; solution 40 ml.
5	red	Incubation buffer	Ready to use solution, 100 ml.
6	white	Substrate buffer	Solution, ready to dissolve the ABTS[1] tablet, 15 ml.
7	white	ABTS substrate tablet	1 piece, sufficient for 15 ml substrate solution
8		Microtiter plate	12 microtiter plate modules (8 wells, each); frame.
			Adhesive cover foils to cover microtiter plate.

2.2 Test principle (sandwich ELISA)



The assay is based on the quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates.

In the first incubation step, anti-histone antibody is fixed adsorptively on the wall of the microtiter plate module. Subsequently, non-specific binding sites on the wall are saturated by treatment with incubation buffer (= blocking solution). During the second incubation step, the nucleosomes contained in the sample bind via their histone components to the immobilized anti-histone antibody. In the third incubation step, anti-DNA-peroxidase (POD) reacts with the DNA-part of the nucleosome. After removal of unbound peroxidase conjugate by a washing step, the amount of peroxidase retained in the immunocomplex is determined photometrically with ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)])*, as a substrate.

2.3 Specificity

Anti histone antibody reacts with the histones H1, H2A, H2B, H3 and H4 of various species, e.g. man, mouse, rat, hamster, cow, opossum, xenopus. Anti-DNA POD antibody binds to single- and double-stranded DNA.

Therefore, the ELISA allows the detection of mono- and oligonucleosomes from various species and may be applied to measure apoptotic cell death in many different cell systems.

2.4 Stability

The kit is stable through the control date printed on the kit when stored at 2-8°C.

3. Preparation of the solutions

The kit contains all the reagents needed and in sufficient amounts for 96 tests. Redistilled water should always be used for reconstitution and dilution purposes.

3.1 Reconstitution of lyophilizates

Anti-Histone (bottle 1)
Reconstitute the lyophilizate in 1 ml redist. water for 10 min and mix thoroughly. Stability of the solution: stable for 2 months, stored at 2-8°C.

Anti-DNA-POD (bottle 2)
Reconstitute the lyophilizate in 1 ml redist. water for 10 min and mix thoroughly. Stability of the solution: stable for 2 months, stored at 2-8°C.

3.2 Preparation of the working solutions

I. Coating solution
Predilute 1 ml coating buffer concentrate (bottle 3) with 9 ml redist. water. Shortly before use, dilute 1 ml anti-histone antibody (bottle 1, reconstituted) with 9 ml coating buffer. Stability of the solution: cannot be stored, prepare immediately before use.

II. Washing solution
Warm up at 15-25°C washing buffer concentrate (bottle 4) and dilute 40 ml in 360 ml redist. water and mix thoroughly. Stability of the solution: stable for 2 months, stored at 2-8°C.

III. Sample solution
Depends on the cell system used and the extent of cell death (see below sample preparation), example: dilute 25 µl sample in 225 µl incubation buffer (bottle 5). Stability of the sample solution: stable for 2 days, stored at 2-8°C.

IV. Conjugate solution
Dilute 1 ml anti-DNA-POD (bottle 2, reconstituted) with 9 ml incubation buffer (bottle 5). Stability of the conjugate solution: cannot be stored, prepare immediately before use.

V. Substrate solution
Dissolve ABTS substrate tablet (bottle 7) in substrate buffer (total volume of bottle 6). The ABTS solution reacts to light on exposure over a longer period. Stability of the solution: stable for 1 month, stored at 2-8°C, protected from light.

4. Samples

4.1 Sample material

Cytoplasmic fractions (lysates) of cell lines, cells *ex vivo* (see sample preparation) or tissue homogenates (18).

4.2. Sample preparation

Dilute the cells with culture medium to obtain a suitable cell concentration. Depending on the cell type and the cell-death-inducing-agent, the cell number per test has to be determined and optimized. The following cellular model system, in particular the cell number per test, is an example for a test procedure. As a model system for cell death, camptothecin (CAM) was used as the apoptosis-inducing drug. The tests were performed with the human myelogenous leukemic cell line HL60 (ATCC: CCL 240) as target cells (14-17).

4.2.1 Induction of cell death (cellular assay)

Dilute exponentially growing HL60 cells with culture medium to obtain a cell concentration of 10^5 cells/ml and transfer into Eppendorf tubes (500 µl/tube = 5×10^4 cells/tube). Thereafter add 500 µl culture medium with different concentrations of CAM (0 µg CAM/ml to 4 µg CAM/ml). Use value 0 µg/ml as a negative control for the cellular assay (= viable, untreated cells). Close tubes loosely to allow further exchange of gas and incubate at 37°C in a CO_2-incubator for 4 h.

4.2.2 Sample processing

After incubation, centrifuge the cells ion an Eppendorf centrifuge at 1500 rpm (= 200 × g) for 5 min. Discard the supernatant and resuspend the cell pellet in 1 ml culture medium. After an other centrifugation step (1500 rpm, 5 min) resuspend the cell pellet with 500 µl incubation buffer (bottle 5) per tube (1×10^5 cell/ml) mix thoroughly and incubate the sample for approx. 30 min at room temperature (= lysis). Centrifuge the lysate at 15000 rpm (= 20000 × g) for 10 min and remove 400 µl of the supernatant (= cytoplasmic fraction) carefully. Do not shake the pellet (= cell nuclei, containing high molecular weight, unfragmentated DNA).

6. Notes

6.1 Background of the immunoassay

Depending on the individual assay conditions, the background value (incubation buffer instead of sample solution) of the immunoassay may vary. Under normal conditions the background is below 100 mU after 15 min substrate reaction.

6.2 Negative control for cell death induction (cellular assay)

Depending on cell culture conditions, each exponentially growing permanent cell culture contains a certain amount of dead cells (normally approx. 3–8%). In the immunoassay, these inherent dead cells in the untreated sample (without cell-death-inducing-agent) will cause a certain absorbance value. Depending on the amount of dead cells, this value may exceed the absorbance value of the immunoassay background.

6.3 Positive control for the ELISA

The negative control of the cellular assay will cause a certain absorbance value in the immunoassay (see 6.2.). Therefore, a positive control for the immunoassay as an additional component is not necessary. However, if an extra positive control for the immunoassay is desired, it can be prepared following this simple procedure:

Centrifuge an aliquot of untreated cells (5×10^4 cells/tube in 500 µl, see section 4.2.1.) at 1500 rpm for 5 min. Discard the supernatant, resuspend the cell pellet in 500 µl hypertonic buffer (10 mM Tris, pH 7.4, 400 mM NaCl, 5 mM $CaCl_2$ and 10 mM $MgCl_2$) and incubate at 37°C for at least 2 h. Thereafter, spin down the cell fragments at 15 000 rpm, remove the supernatant carefully, dilute the supernatant 1:5 with incubation buffer (bottle 5) und use this sample as a positive control for the immunoassay.

6.4 Detection limit

The exact detection limit of dying/dead cells in a particular sample strongly depends on the kinetic of cell death, the cytotoxic agent used and the amount of affected cells in the total cell population. Using HL60/CAM as a cellular model system for cell death, the immunoassay allows the specific detection of mono- and oligonucleosomes in the cytoplasmic fraction of 5×10^2 cells/ml (= 50 cell equivalents/well) (see fig. 2).

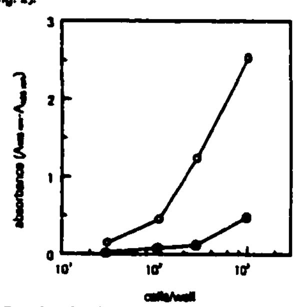


Fig. 2: Detection of nucleosomes in cytoplasmic fractions at different cell concentrations. HL60 cells were cultured at different cell concentrations with CAM (2 µg/ml) or without CAM for 4 h at 37°C. After cell lysis the cytoplasmic fractions of the samples were prediluted 1:10 with incubation buffer and tested in the immunoassay. Substrate reaction time: 10min (○ with CAM, ● without CAM).

7. References

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E-mail Adress	Country
argentina.biochem@roche.com	Argentina
biochem.au@roche.com	Australia
Gerhard.Muehlbauer@roche.com	Austria
biochem.be@roche.com	Belgium
biochem.ca@roche.com	Canada
biochem.cn@roche.com	China
[illegible]	Cyprus
[illegible]	Czech Republic
[illegible]	Finland
biochem.fr@roche.com	France
[illegible]	Germany
[illegible]	Iran
[illegible]	Israel
[illegible]	Italy
[illegible]	Japan
[illegible]	Korea
[illegible]	Netherlands
biochem.nz@roche.com	New Zealand
[illegible]	Norway
biochem.pt@roche.com	Portugal
biochem.sg@roche.com	Singapore
[illegible]	South Africa
biochem.es@roche.com	Spain
biochem.se@roche.com	Sweden
Biocheminfo.CH@roche.com	Switzerland
uk.biochem@roche.com	United Kingdom
biochemts.us@roche.com	USA
biochemts.row@roche.com	All other countries

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Predilute the resulting supernatant 1:10 with incubation buffer (≈1 × 10^6 cell equivalents/ml) and detect the nucleosomes in the sample by immunoassay (see below section 5.).

It is recommended that the samples are stored in aliquots at −15 to −25°C if they cannot be tested on the same day or, at the latest, one day later.

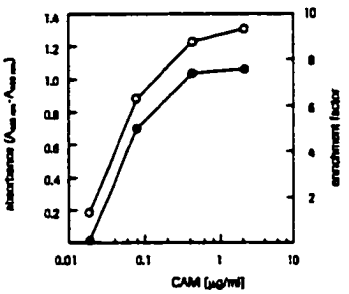

Fig. 1: Enrichment of nucleosomes in the cytoplasm of cells treated with camptothecin. HL60 cells were exposed for 4 h at 37°C to different CAM concentrations as described above. After cell lysis and centrifugation, the cytoplasmic fractions were prediluted 1:10 with incubation buffer and tested for nucleosomes by ELISA (≈ 1 × 10^6 cell equivalents/ml = 1 × 10^5 cell equivalents/well; for definition of cell equivalents see section 5). Substrate reaction time: 15 min (○ absorbance [10^{-3}], ● enrichment factor).

5. Working procedure of the ELISA

Elapsed time: approx. 5–6 h.

Temperature: 15–25°C.

It is recommended that the cytoplasmic fractions are diluted with incubation buffer to obtain a sample solution corresponding to approx. 10^3–10^4 cell equivalents/ml. The amount of 10 × cell equivalents/ml is defined as the volume of cell lysate which corresponds to 10 × cells/ml.

For the immunoassay, it is recommended that the samples be measured and titrated in duplicate. Titrate also the negative control of the cellular assay (= viable cells). This allows calculation of the enrichment factor (see below).

5.1 Coating of the MTP-modules with anti-histone

Pipette 100 µl coating solution (I) into each well of the MTP-modules; cover MTP-modules tightly with the adhesive cover foil included and incubate for 1 h at room temperature (alternatively overnight at 2–8°C).

5.2 Recoating

Remove coating solution thoroughly by tapping or suction. Pipette 200 µl incubation buffer (bottle 5) into each well of the MTP-modules. Cover MTP-modules tightly with the adhesive cover foil included and incubate for 30 min at 15–25°C.

5.3 Washing

Remove solution thoroughly by tapping or suction. Rinse wells three times with 250–300 µl washing solution (II) per well and remove washing solution carefully.

5.4 Incubation with sample solution

Pipette 100 µl of sample solution (III) into each well of the MTP-modules. For determination of the background of the immunoassay, pipette 100 µl per well of incubation buffer (bottle 5) into two wells. Cover MTP-modules tightly with the adhesive cover foil included and incubate for 90 min at 15–25°C.

5.6 Washing

As described under 5.3.

5.6 Incubation with anti-DNA-peroxidase

Pipette 100 µl of conjugate solution (IV) into each well of the MTP-modules, except the blank position. Cover MTP-modules tightly with the included adhesive cover foil and incubate for 90 min at room temperature.

5.7 Washing

As described under 5.3.

5.8 Substrate reaction

Pipette 100 µl of substrate solution (V) into each well of the MTP-modules and incubate on a plate shaker at 250 rpm until the color development is sufficient for a photometric analysis (approx. after 10–20min).

5.9 Measurement

Homogenize the content of the wells by careful tapping at the MTP edges and measure at 405 nm against substrate solution (V) as blank.

5.10 Interpretation

Average the values from the double absorbance measurements of the samples. Subtract the background value (see below) of the immunoassay from each of these averages. Calculate the specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values using the following formula:

$$\frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding control (viable cells)}} = \text{enrichment factor}$$

mU = absorbance [10^{-3}]

Samples with values exceeding the measurement range of the photometer ("over") should be diluted and run again; the corresponding control sample (viable cells) has to be diluted by the same factor. Please note this dilution factor when calculating the enrichment factor. Alternatively, the substrate reaction time can be decreased.

APPENDIX 2. INDIVIDUAL CALF TEMPERATURE PROFILES FROM CHAPTER 5.

In this experiment, healthy weaned heifer calves, approximately 6 months old, were challenged intrabronchially with approximately 1 x 10^8 colony forming units/ml of *Pasteurella haemolytica*. Approximately 20 hours after challenge, calves were treated with danofloxacin mesylate (180 mg/ml) at a dosage of 6 mg/kg subcutaneously in the left lateral neck, tilmicosin (300 mg/ml) at a dosage of 10 mg/kg subcutaneously in the left lateral neck, or saline (0.9%) in a volume equal to a dose of danofloxacin subcutaneously in the left lateral neck. Eight animals were also used as controls, and were neither challenged nor treated. Core body temperatures were recorded continuously over the entire experiment for each animal using an intravaginal radiotransmitter. The graphs in this appendix represent body temperatures for each animal in the experiment, with outlier data points removed according to the description in Chapter 5. The vertical lines in the graph represent the time of challenge and the time of treatment.

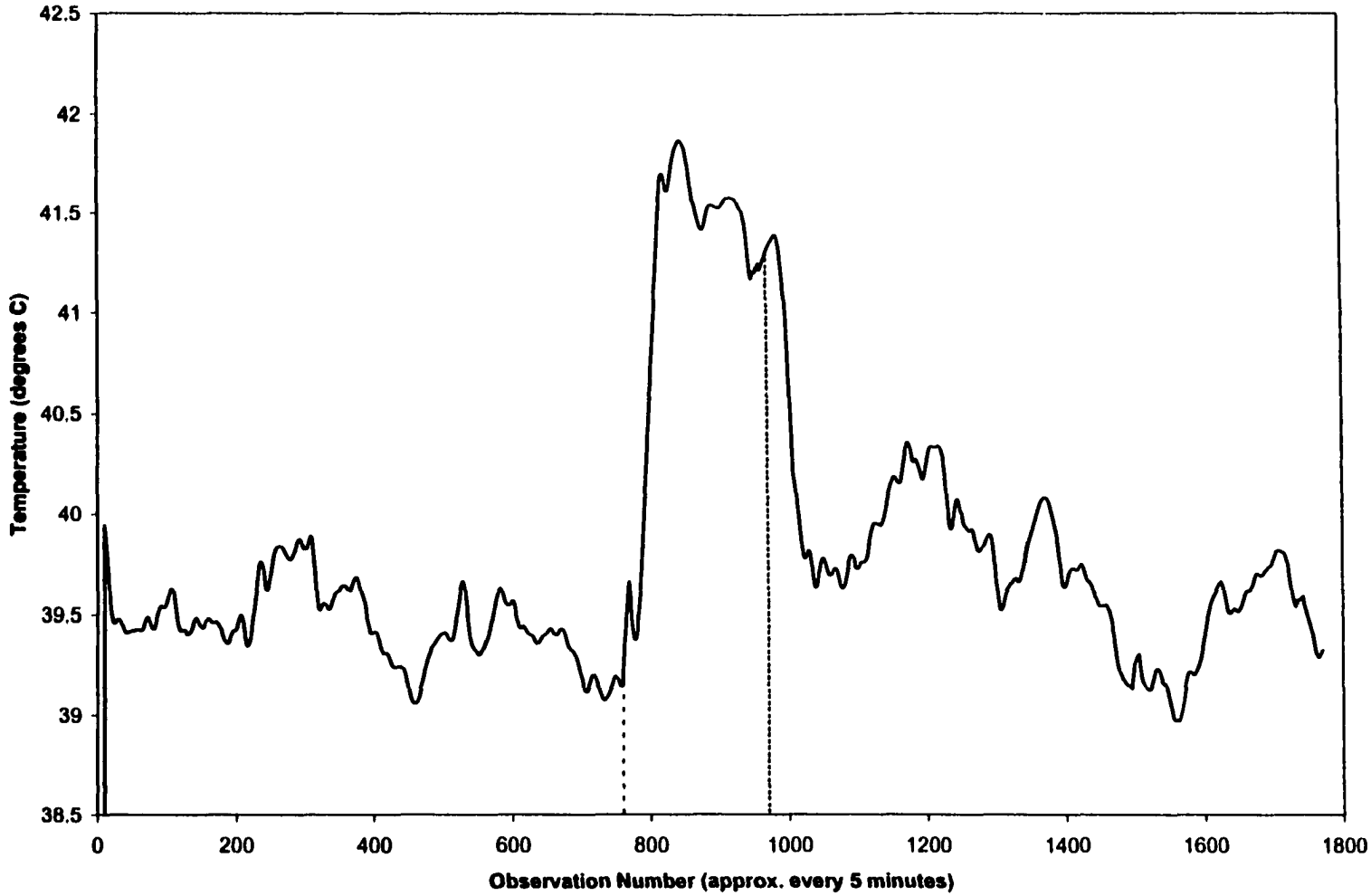

Figure 1. Animal No. 4; treated with danofloxacin

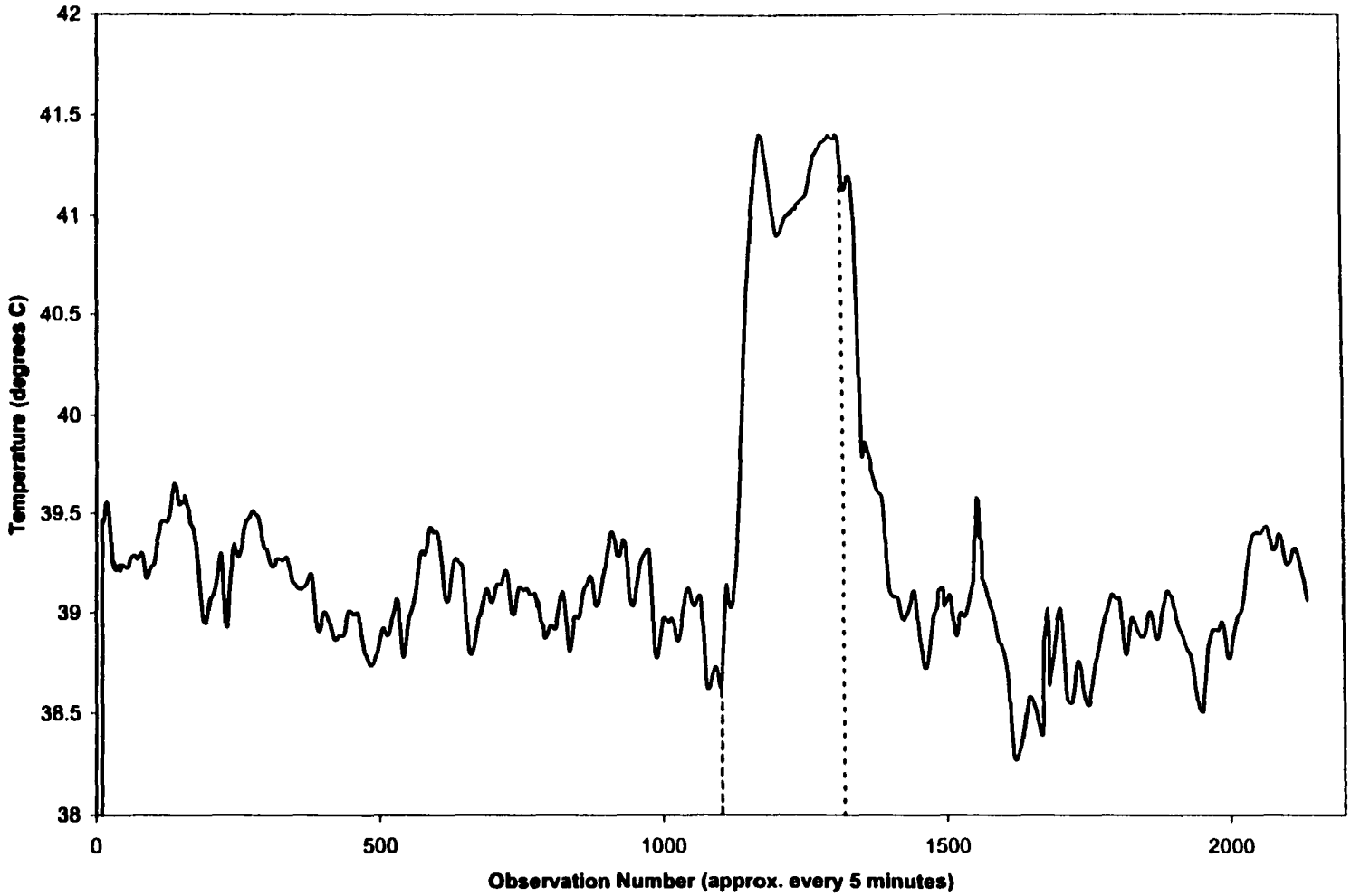

Figure 2. Animal No. 5; treated with danofloxacin

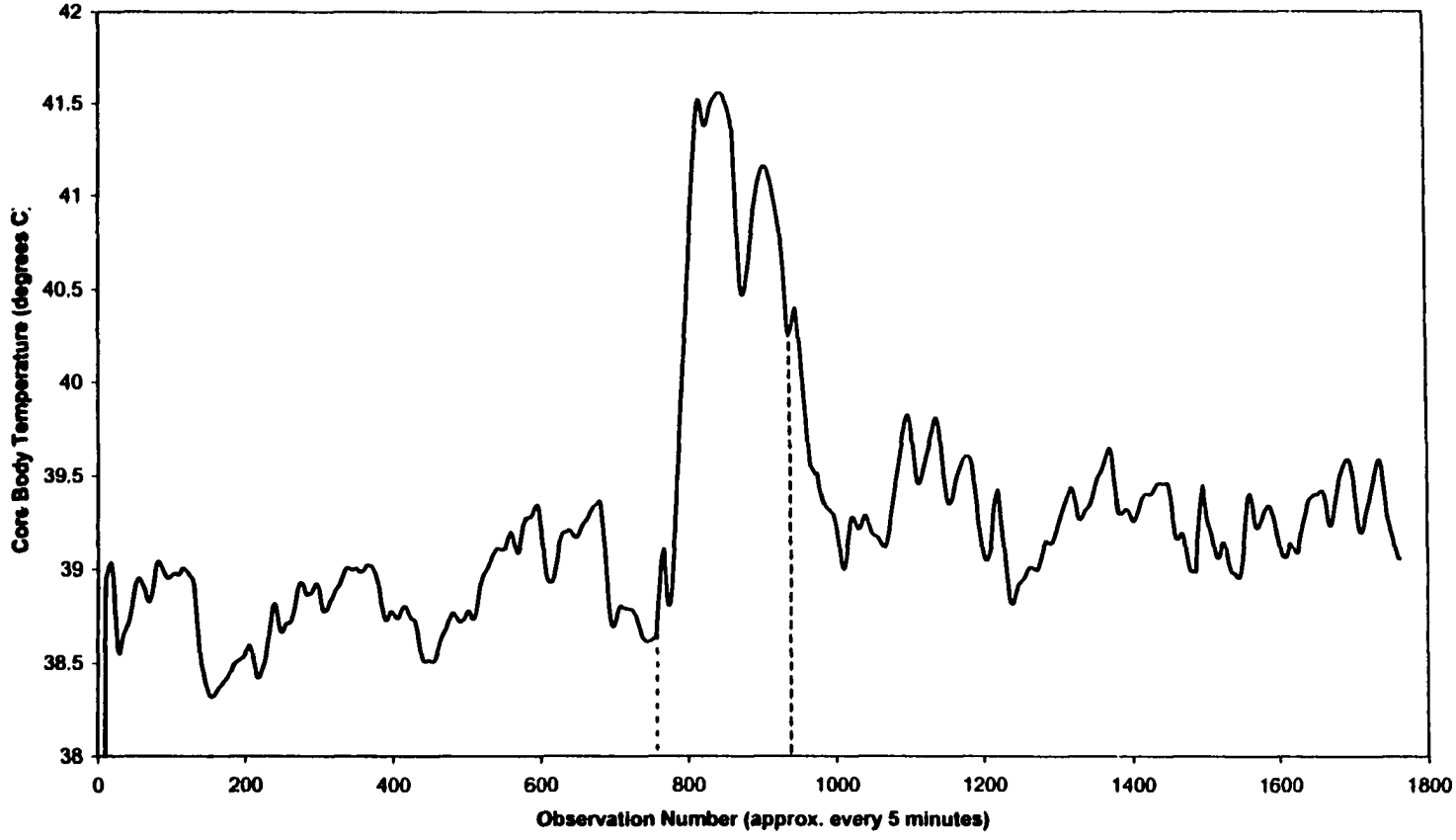

Figure 3. Animal No. 6; treated with saline

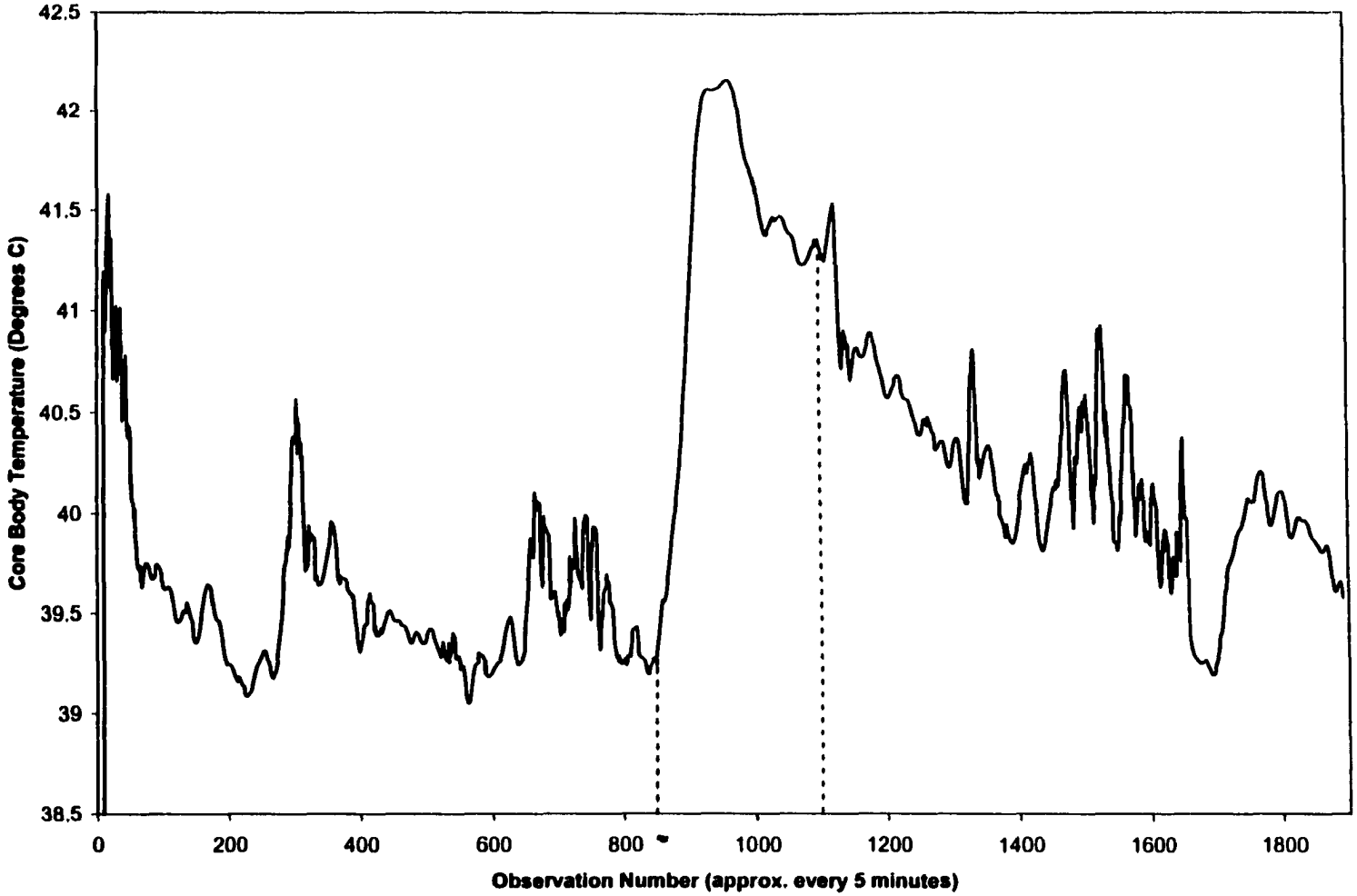

Figure 4. Animal No. 7; treated with saline

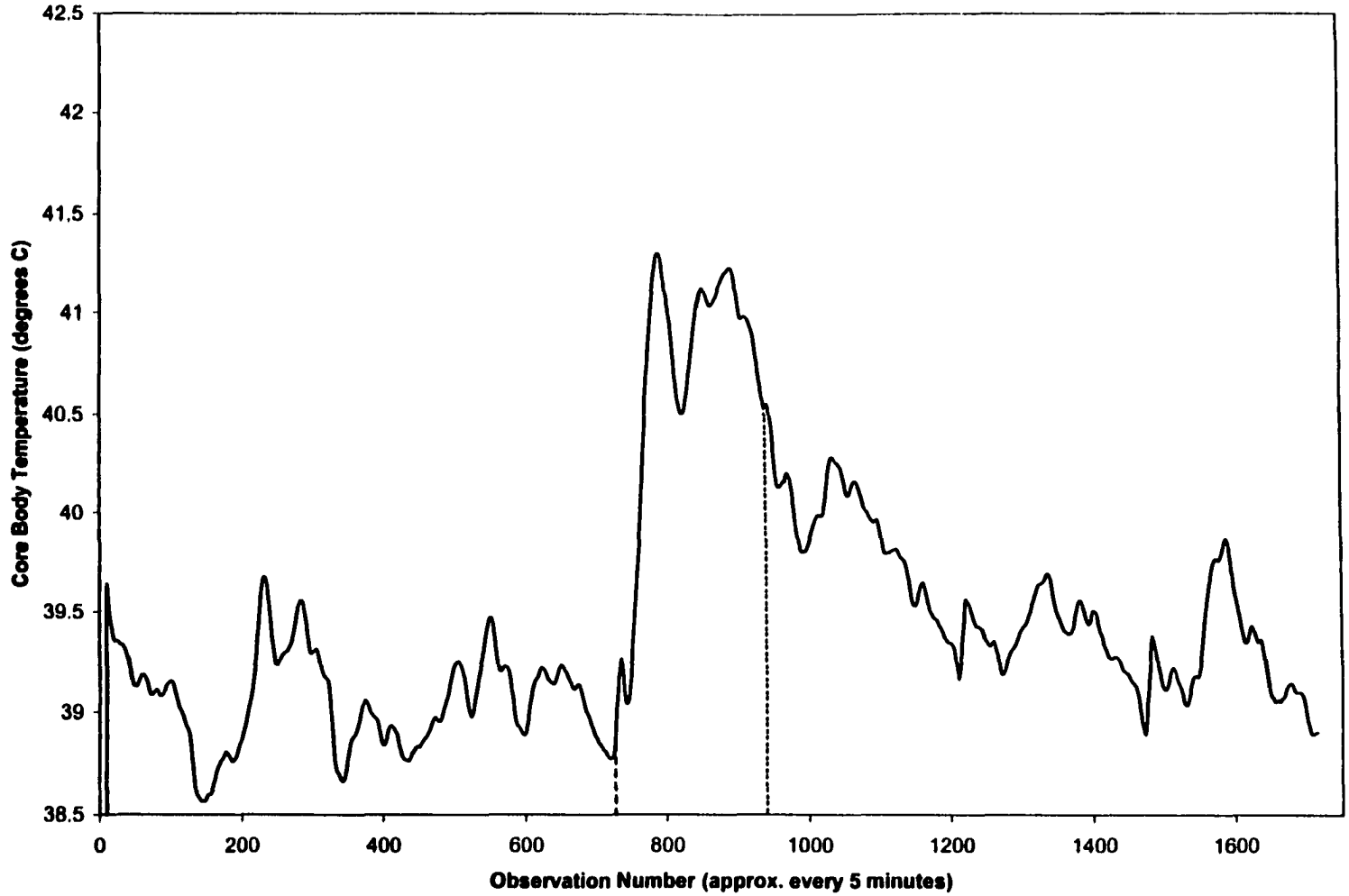

Figure 5. Animal No. 8; treated with danofloxacin

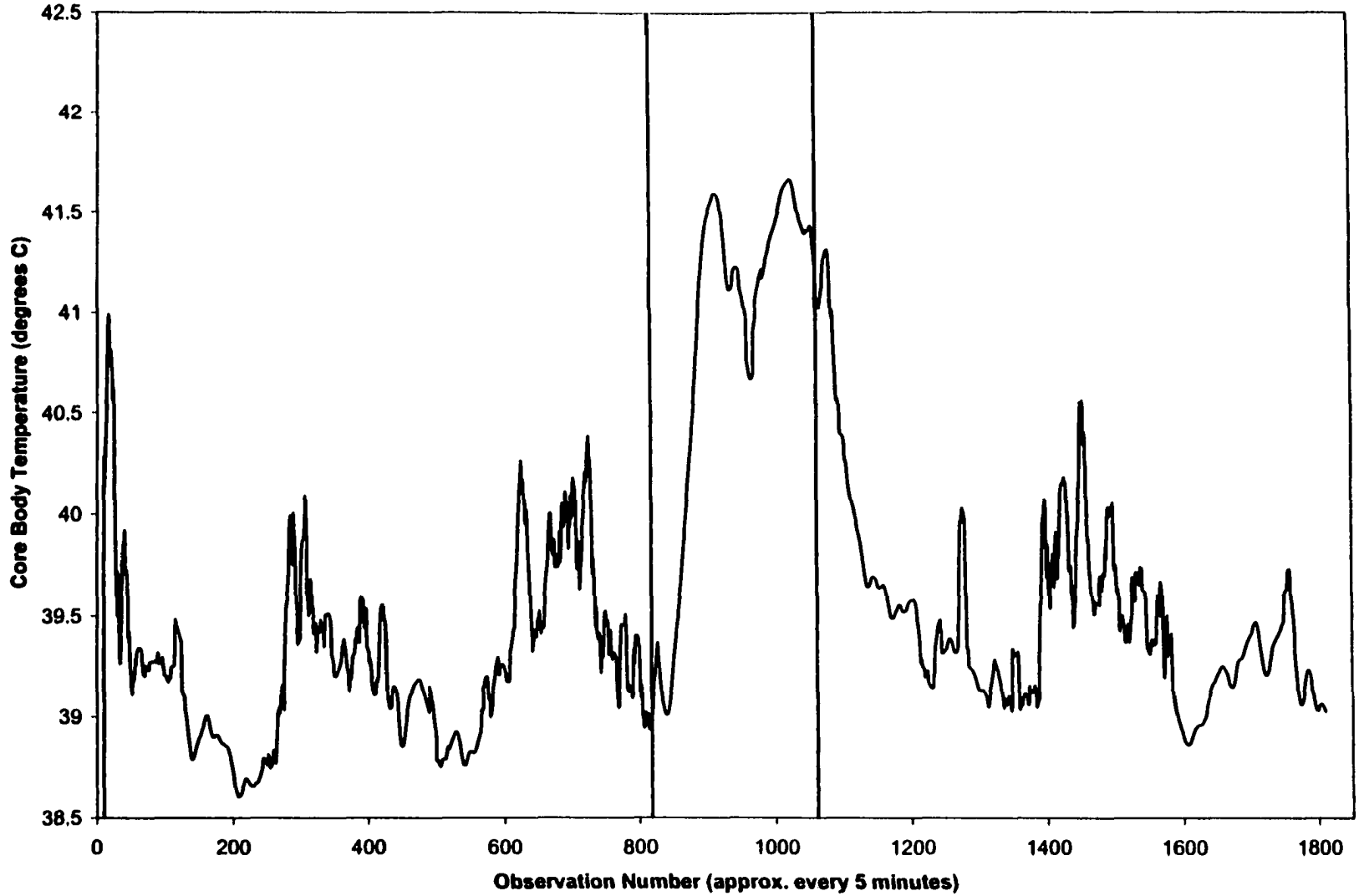

Figure 6. Animal No. 14; treated with danofloxacin

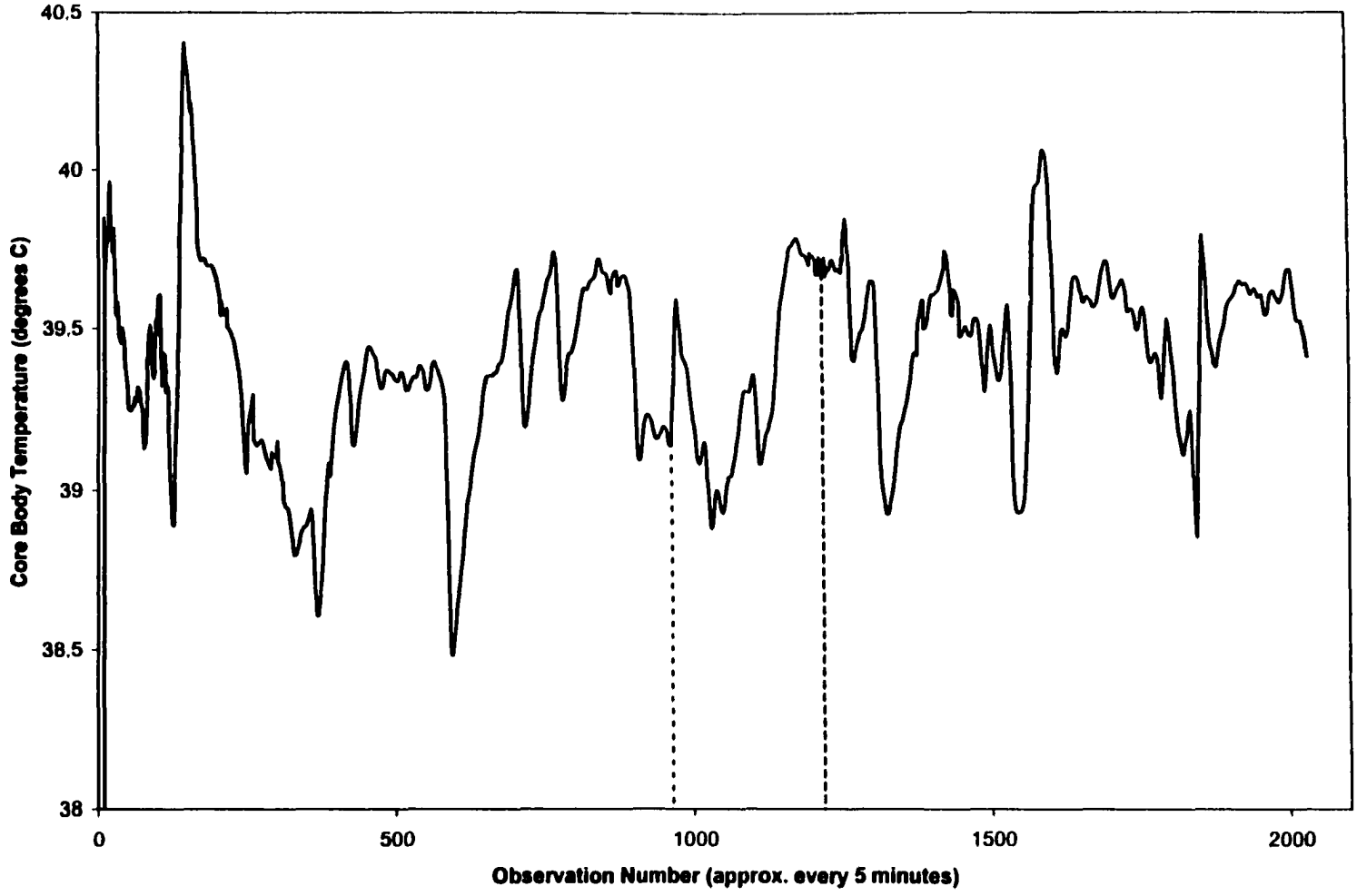

Figure 7. Animal No. 15; non-challenged

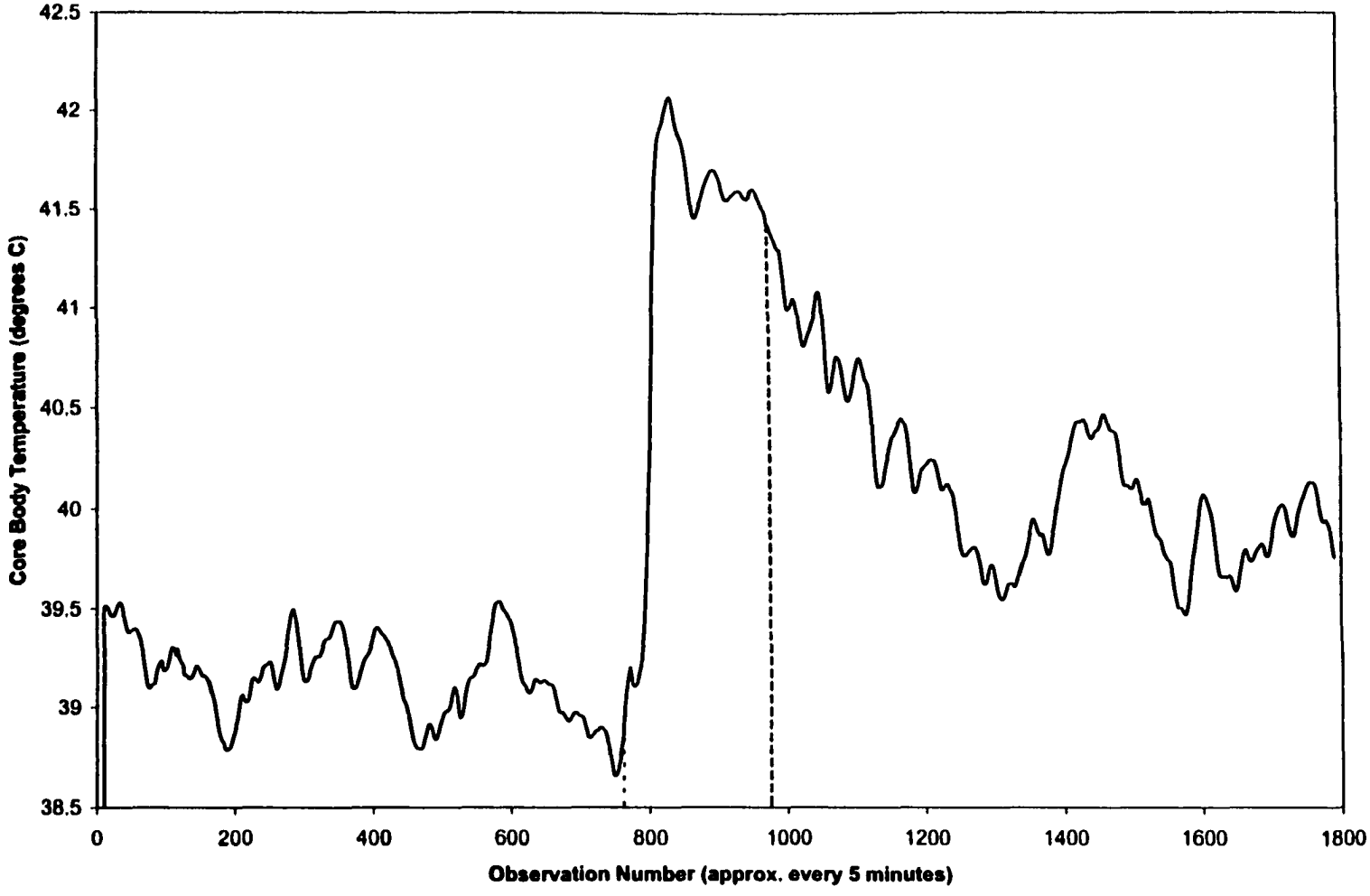

Figure 8. Animal No. 17; treated with saline

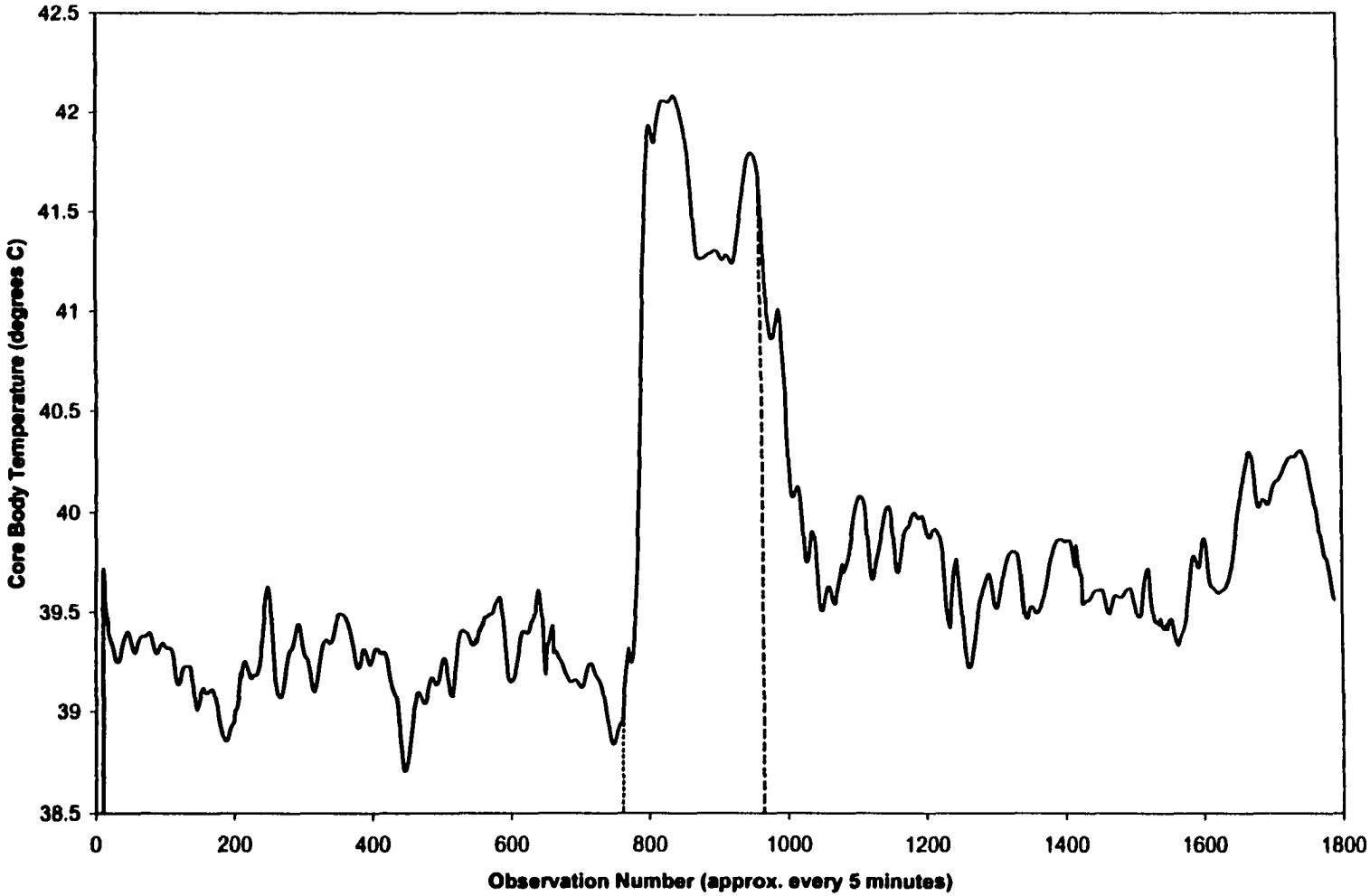

Figure 9. Animal No. 20; treated with danofloxacin

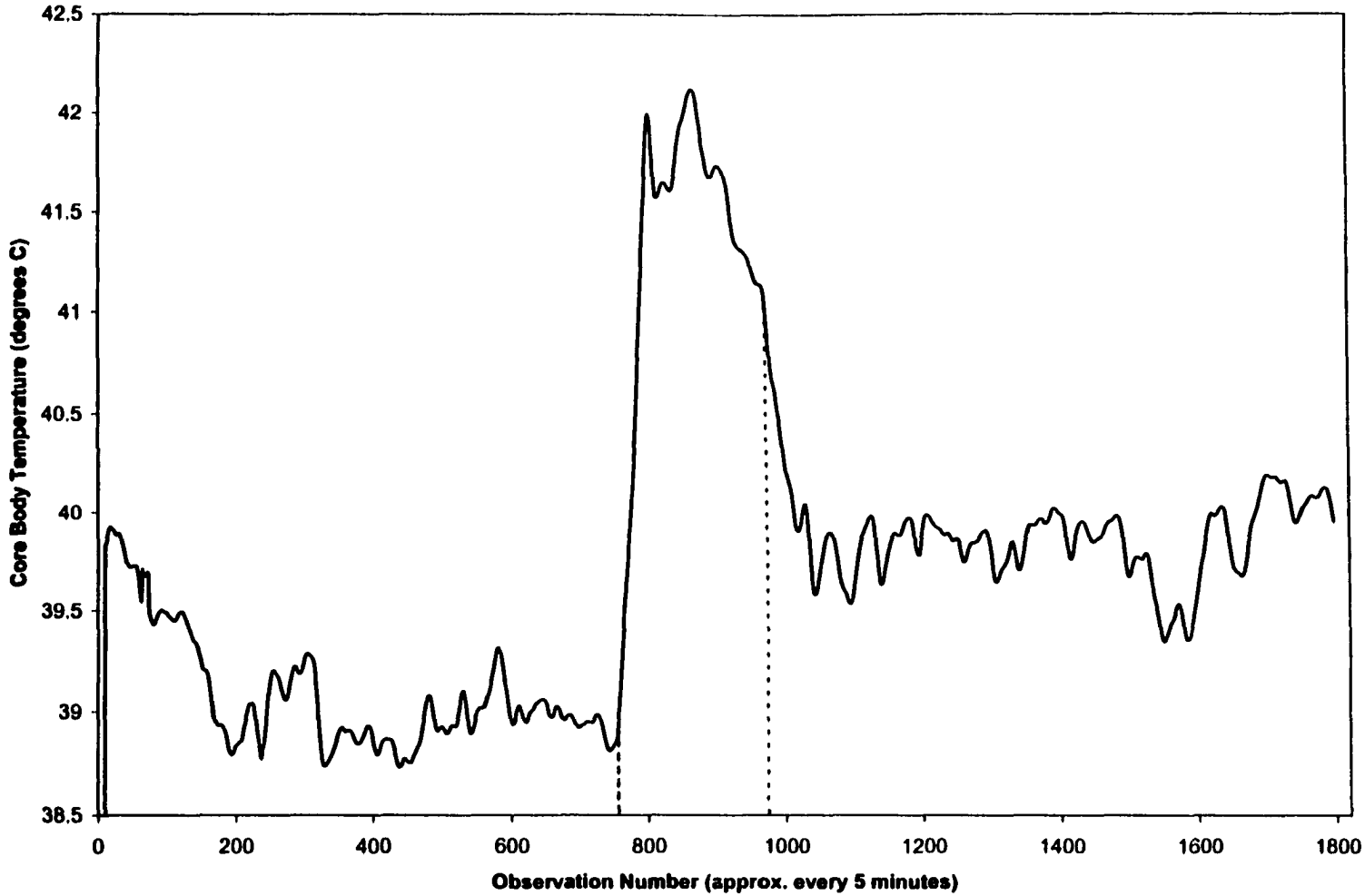

Figure 10. Animal No. 26; treated with saline

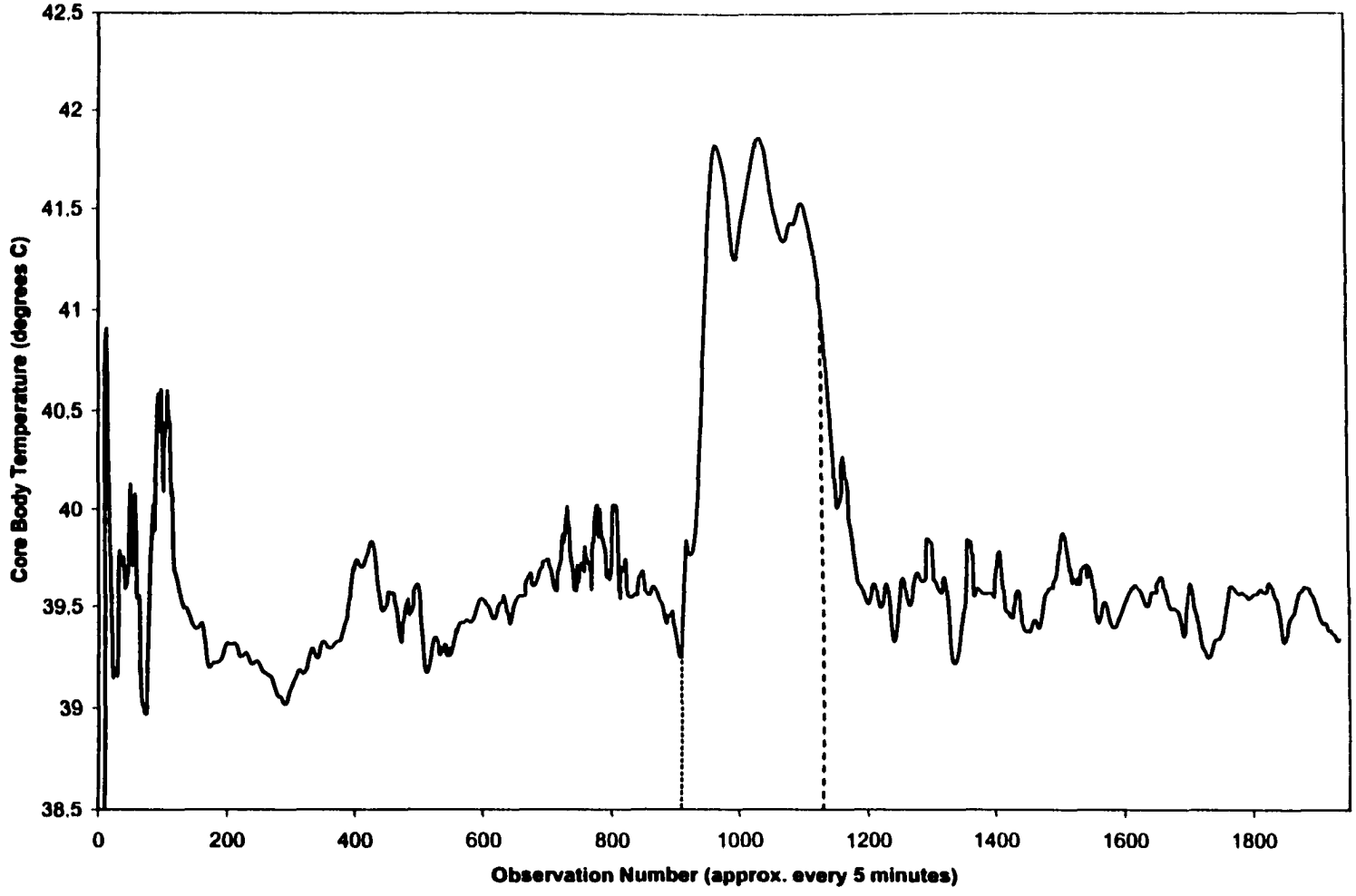

Figure 11. Animal No. 30; treated with saline

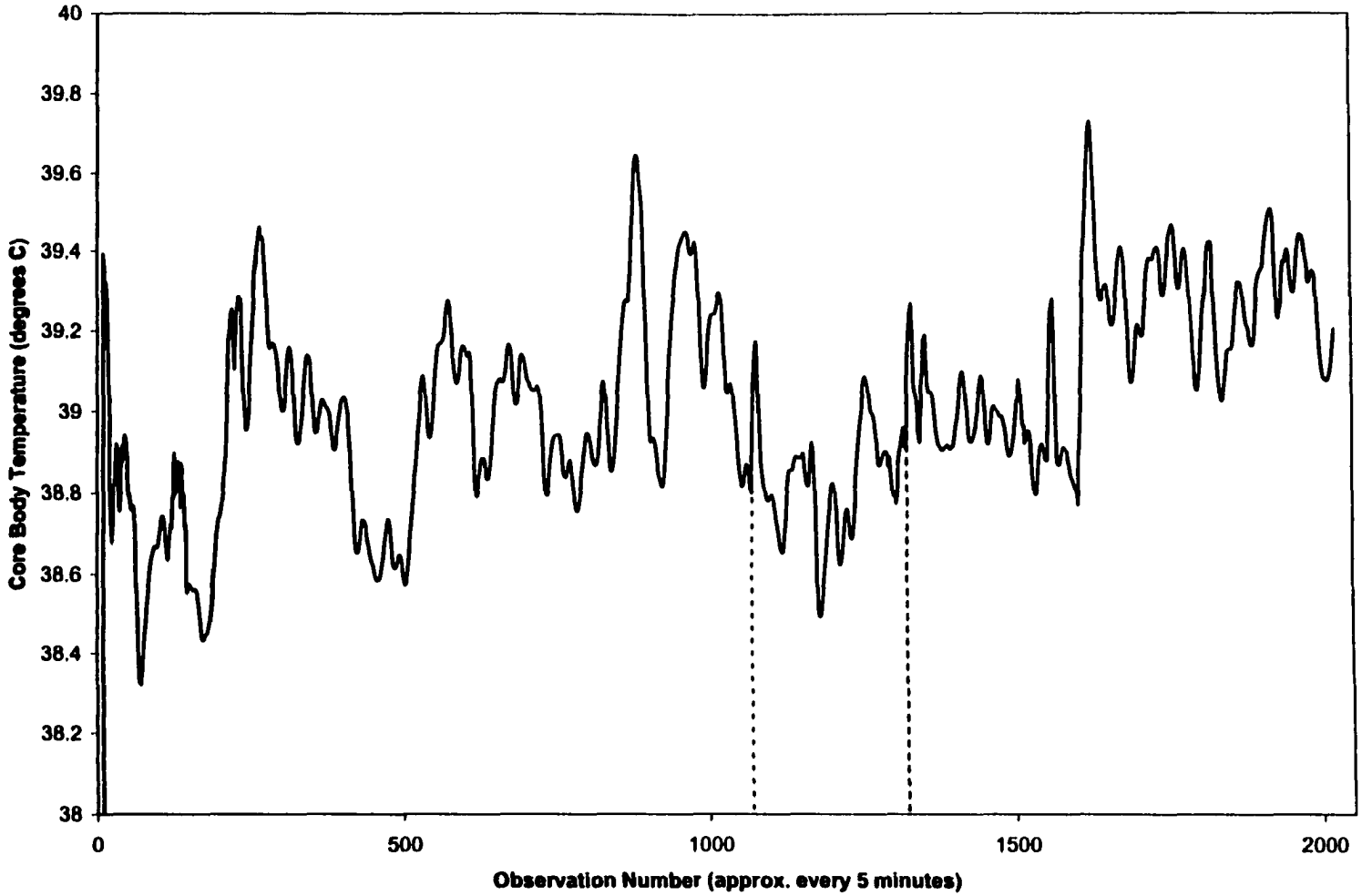

Figure 12. Animal No. 31; non-challenged

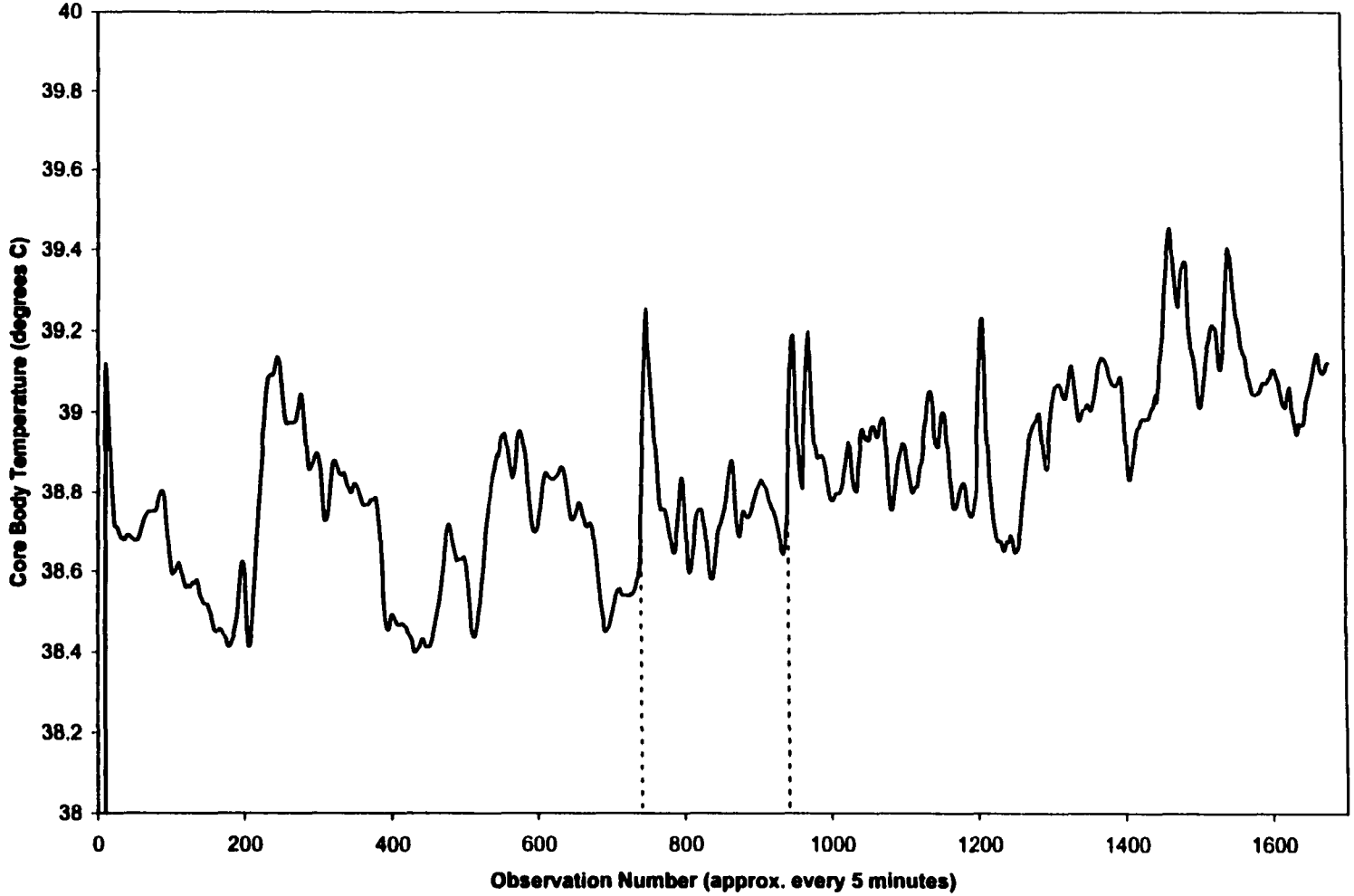

Figure 13. Animal No. 32; non-challenged

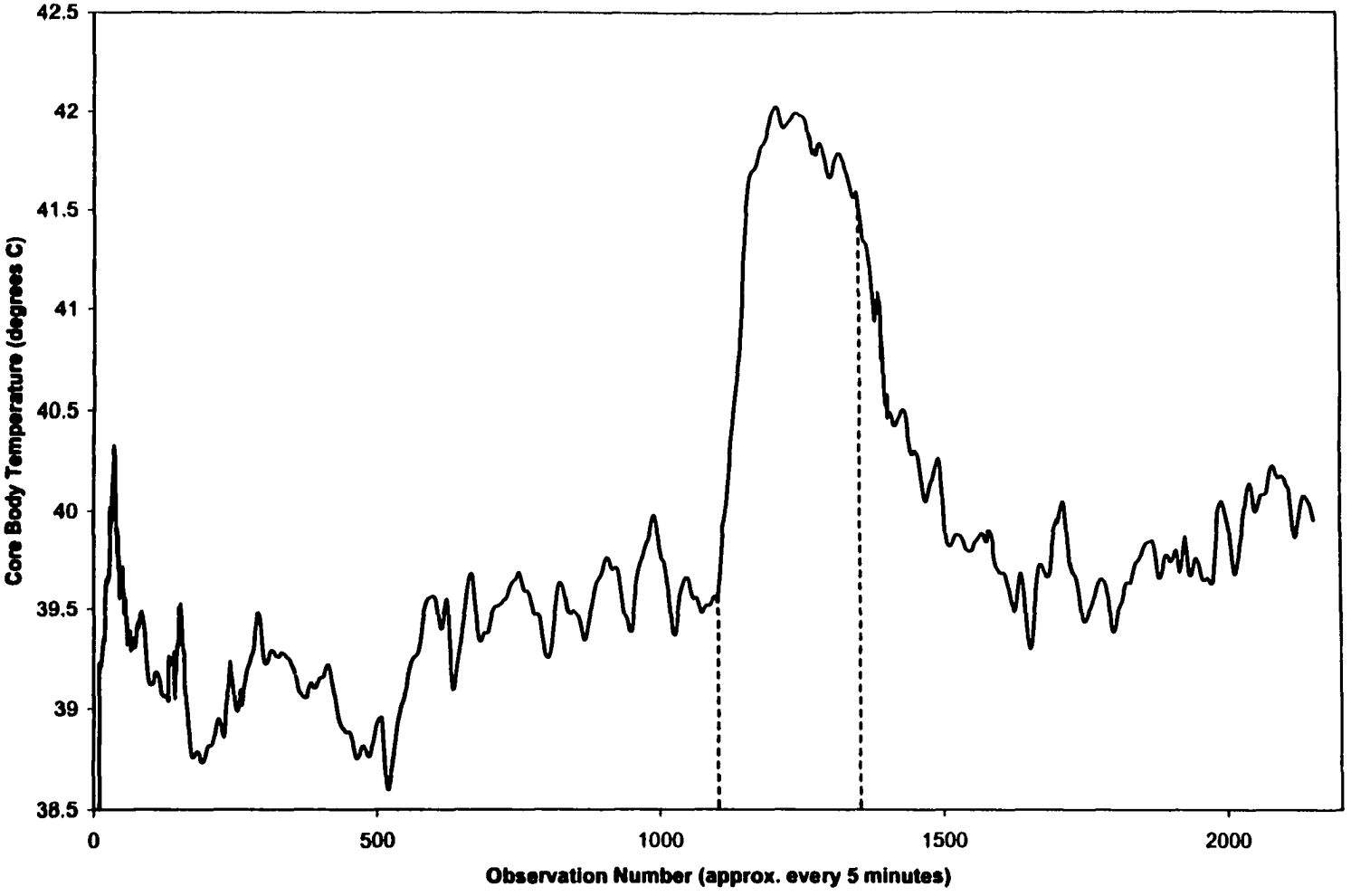

Figure 14. Animal No. 33; treated with saline

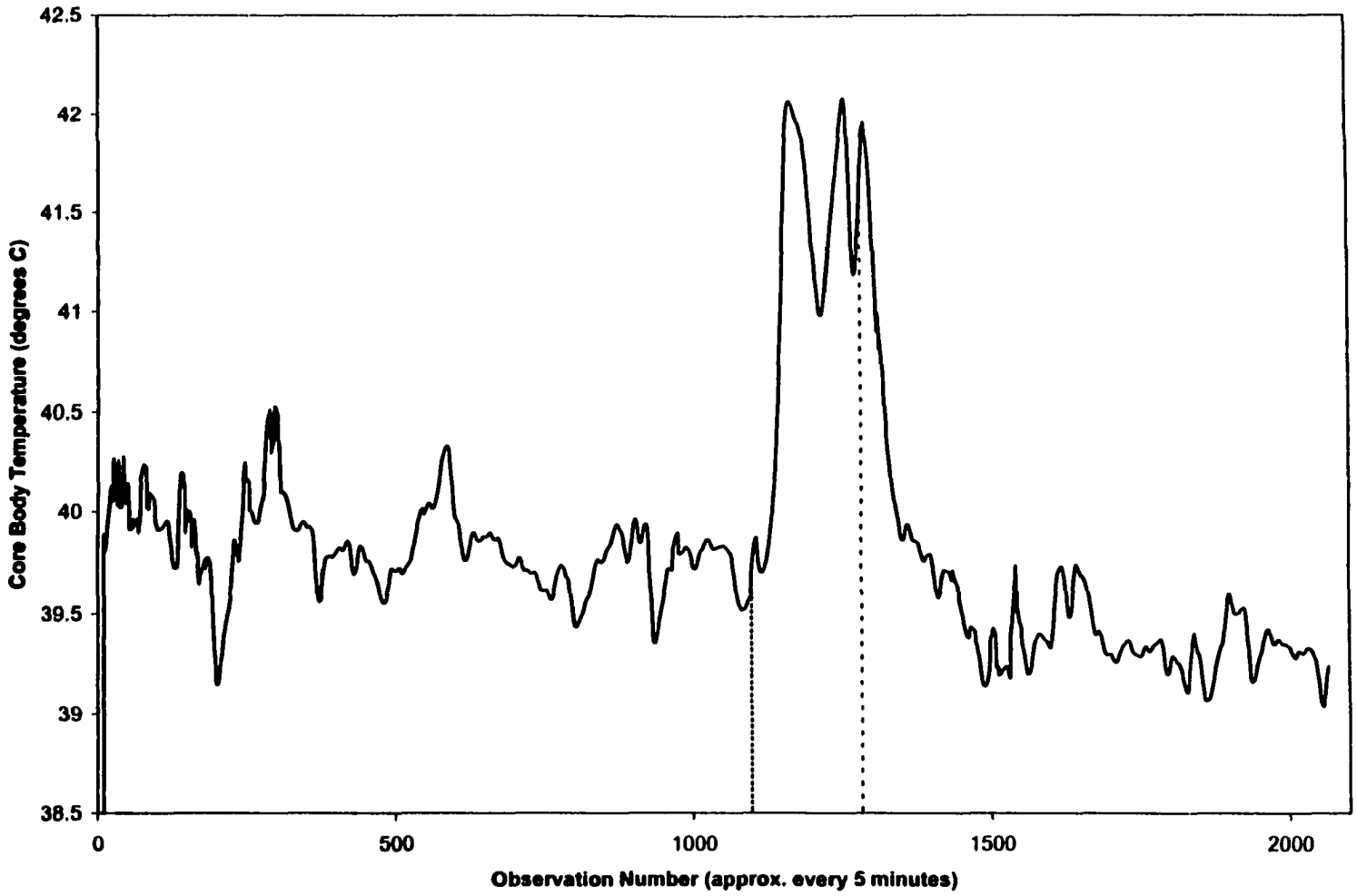

Figure 15. Animal No. 34; treated with tilmicosin

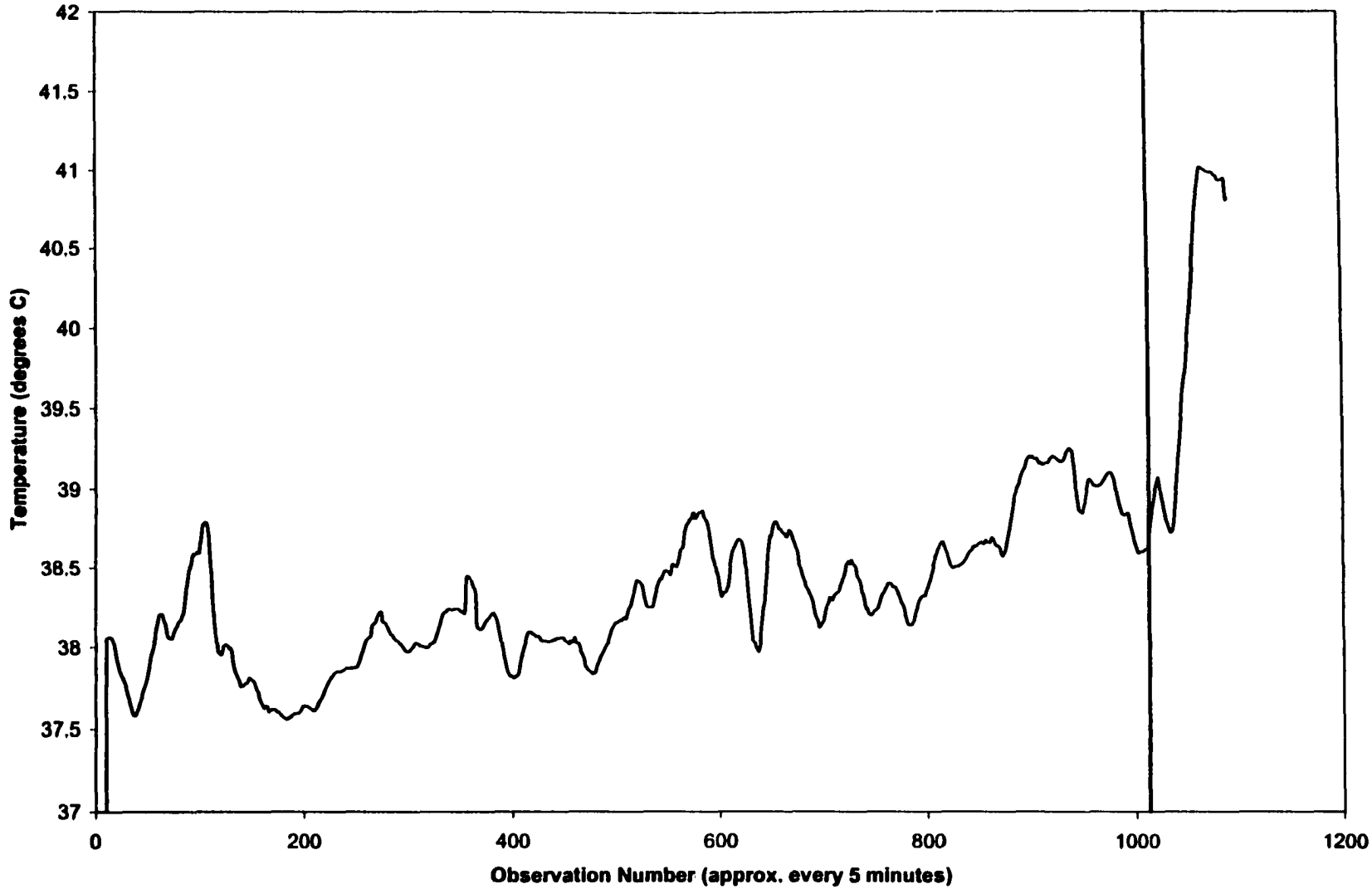

Figure 16. Animal No. 35; treated with tilmicosin

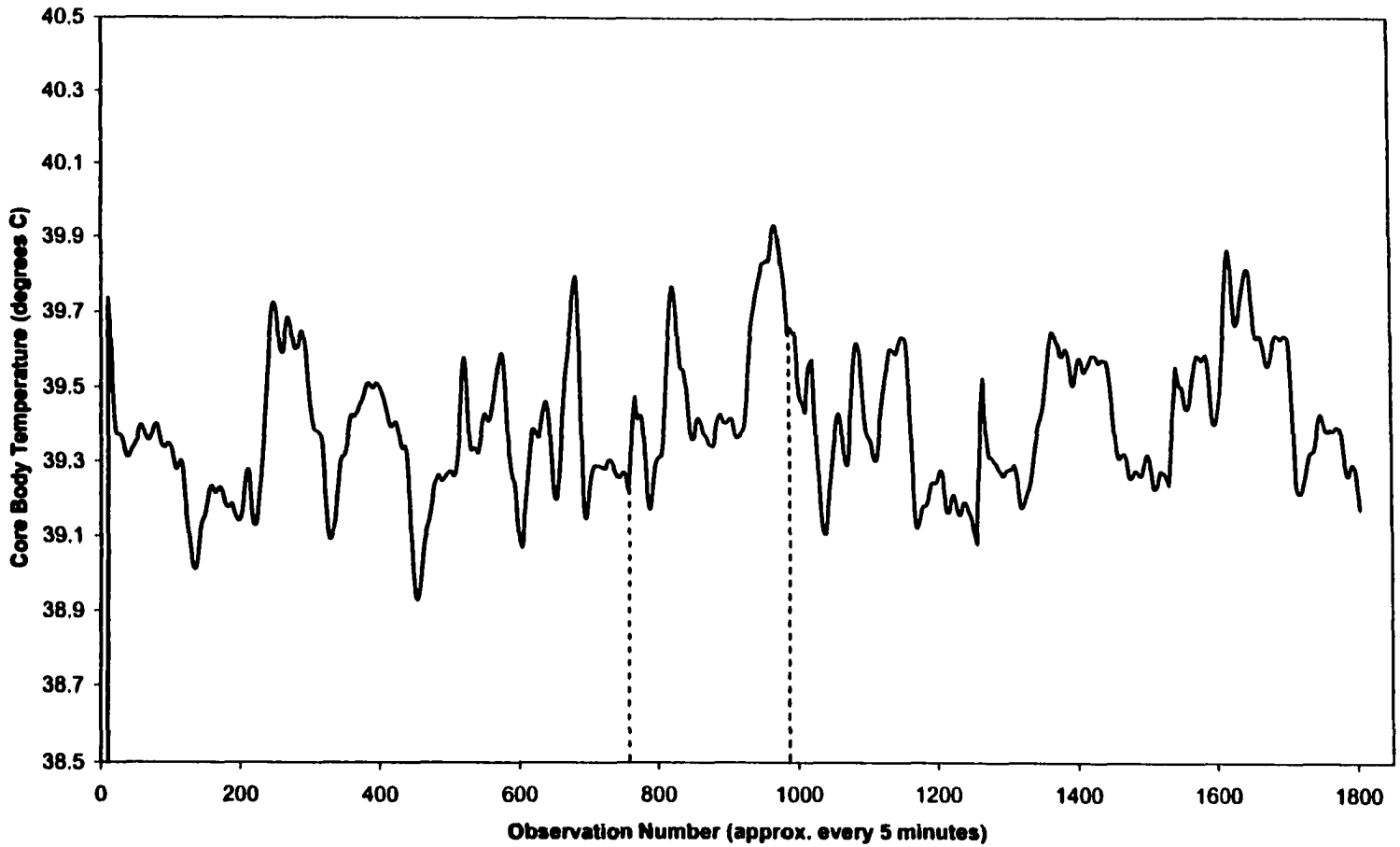

Figure 17. Animal No. 37; non-challenged

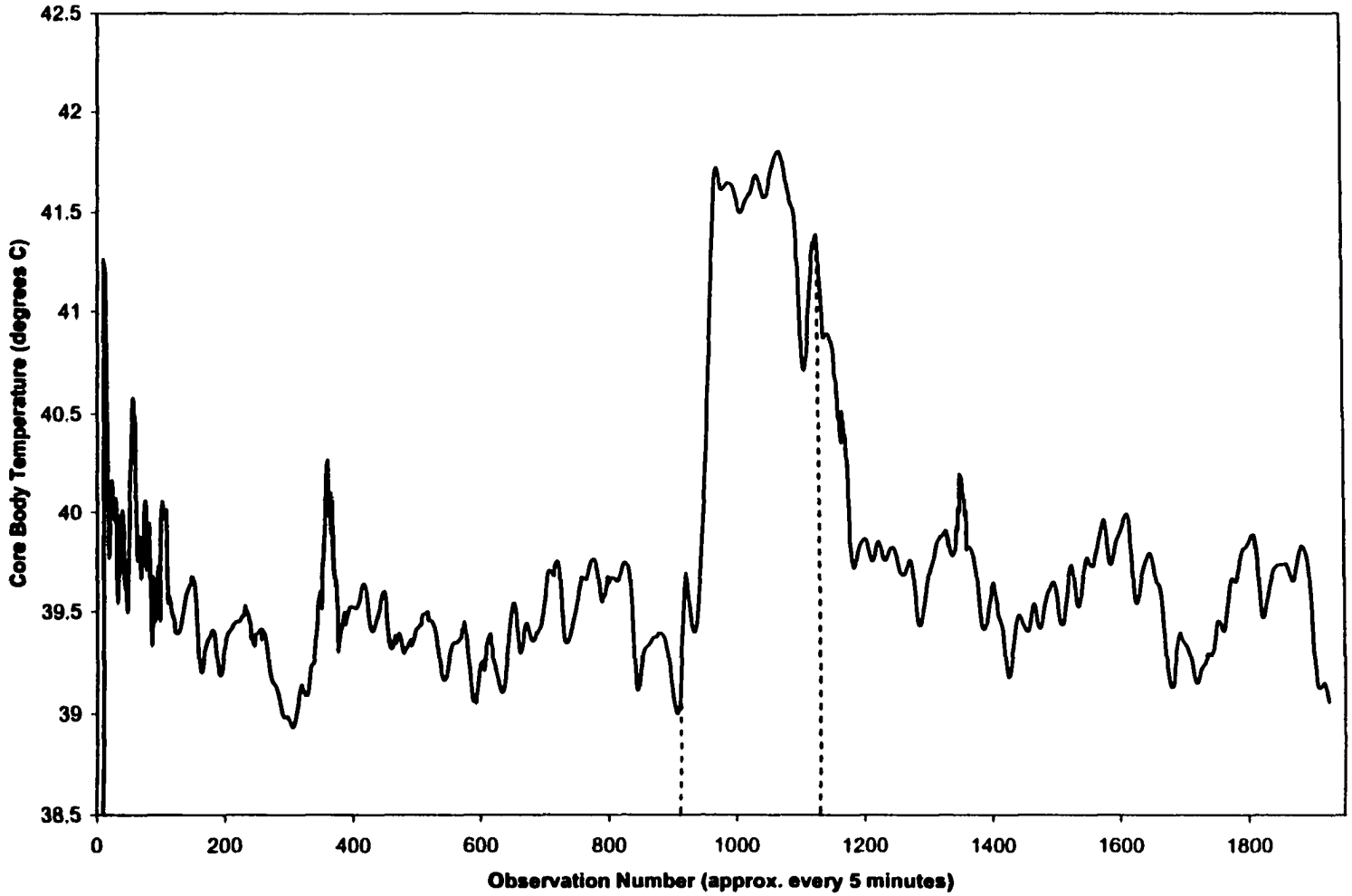

Figure 18. Animal No. 38; treated with danofloxacin

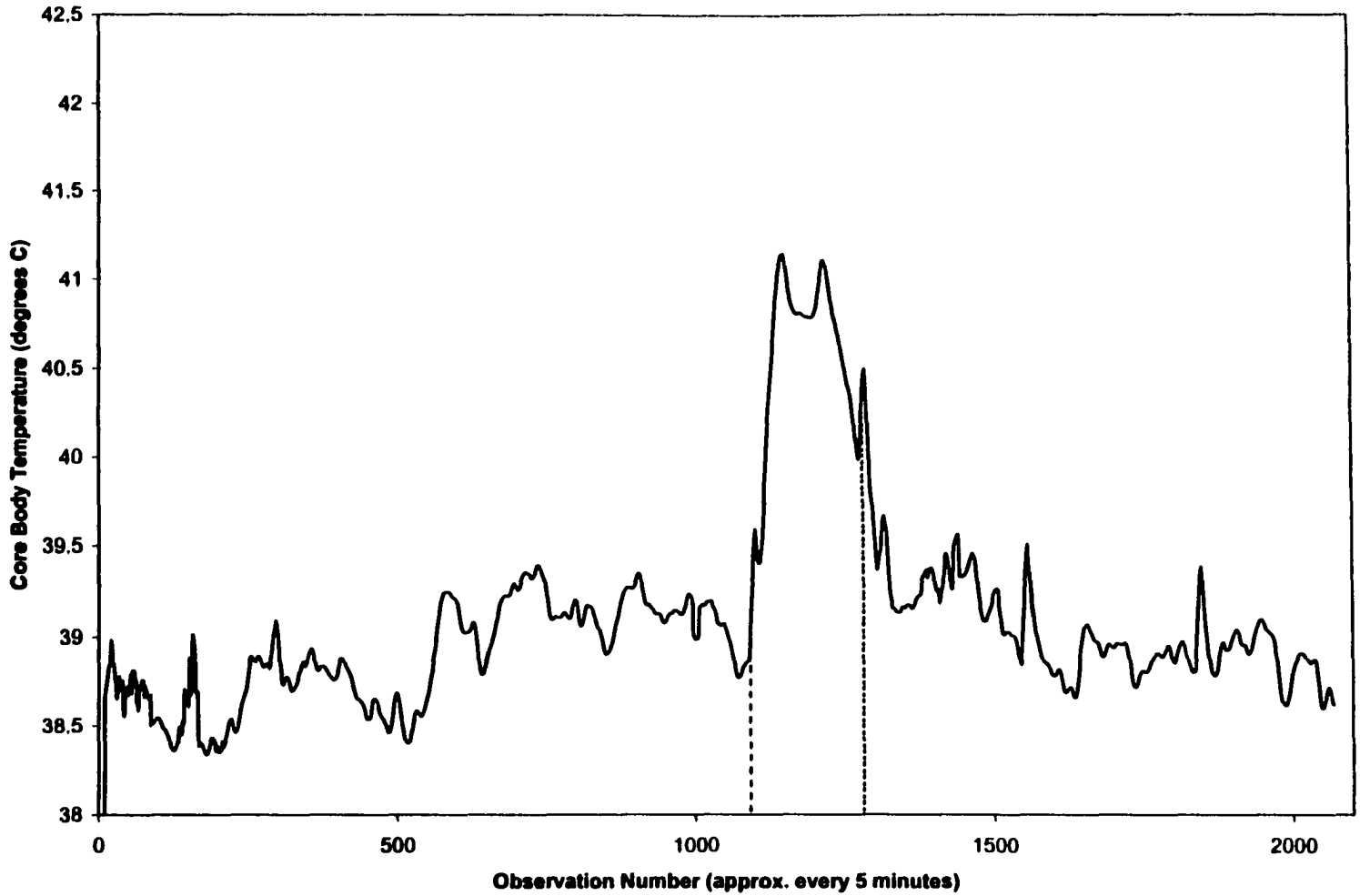

Figure 19. Animal No. 39; treated with tilmicosin

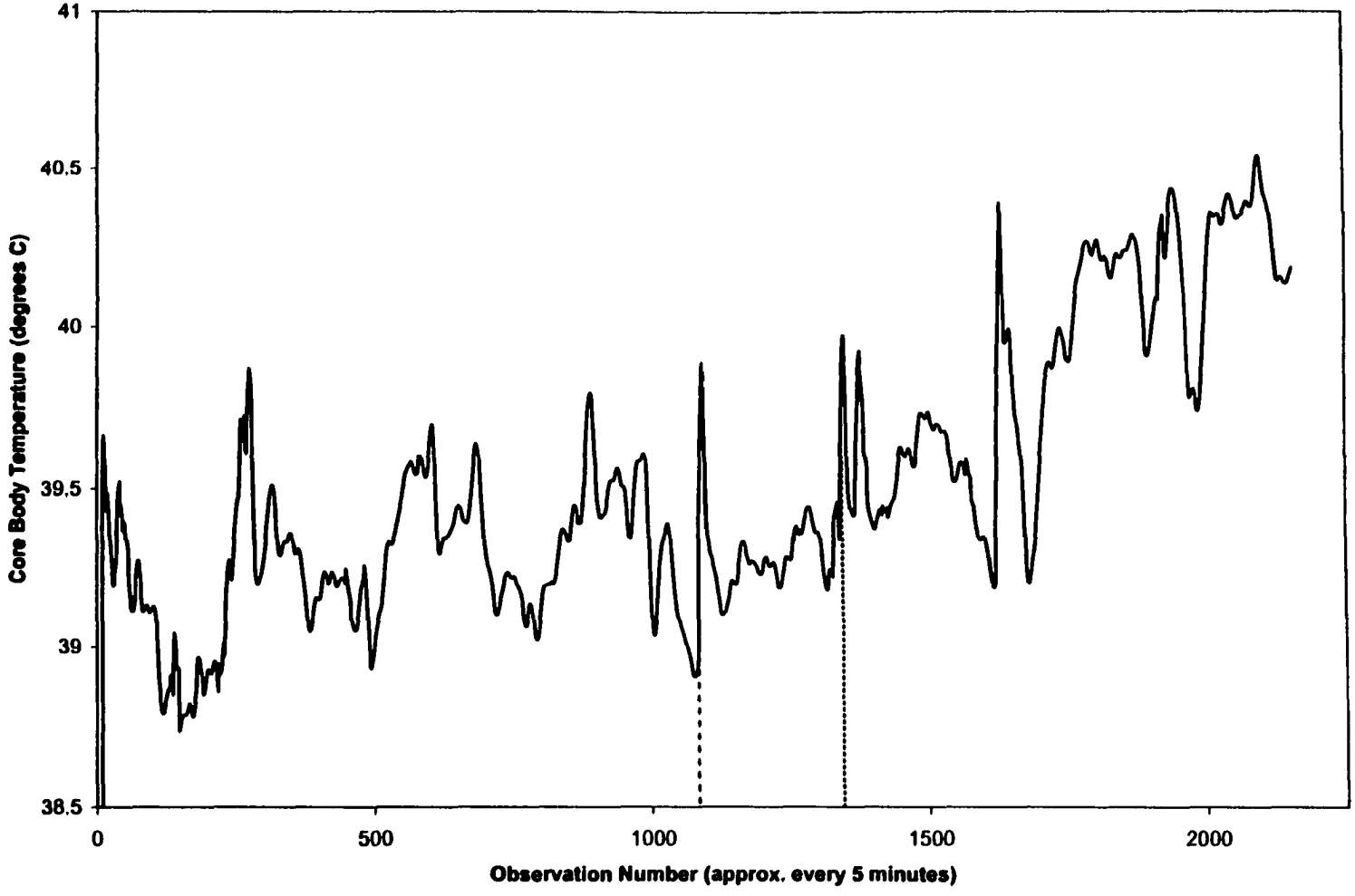

Figure 20. Animal No. 40; non-challenged

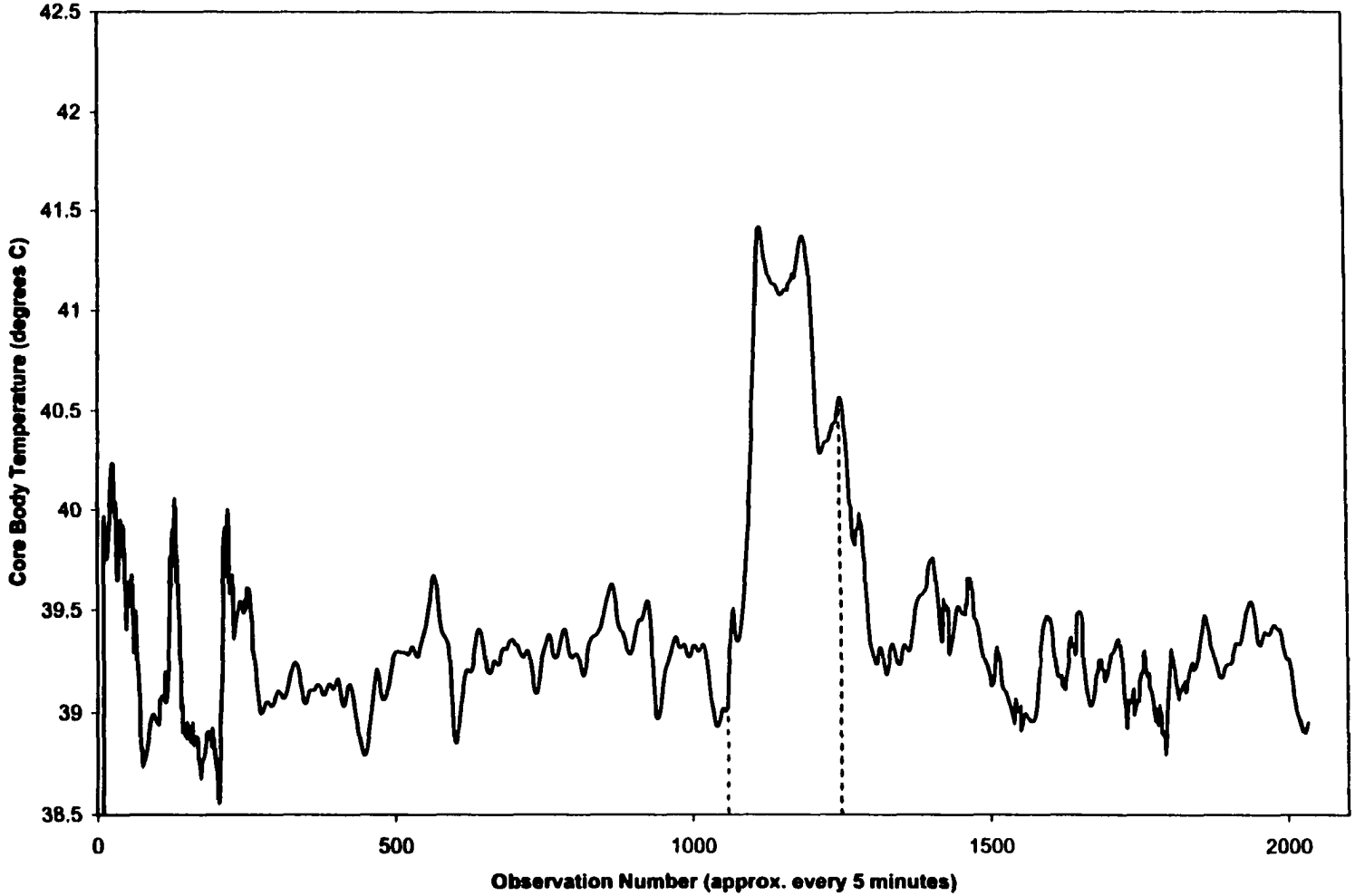

Figure 21. Animal No. 42; treated with tilmicosin

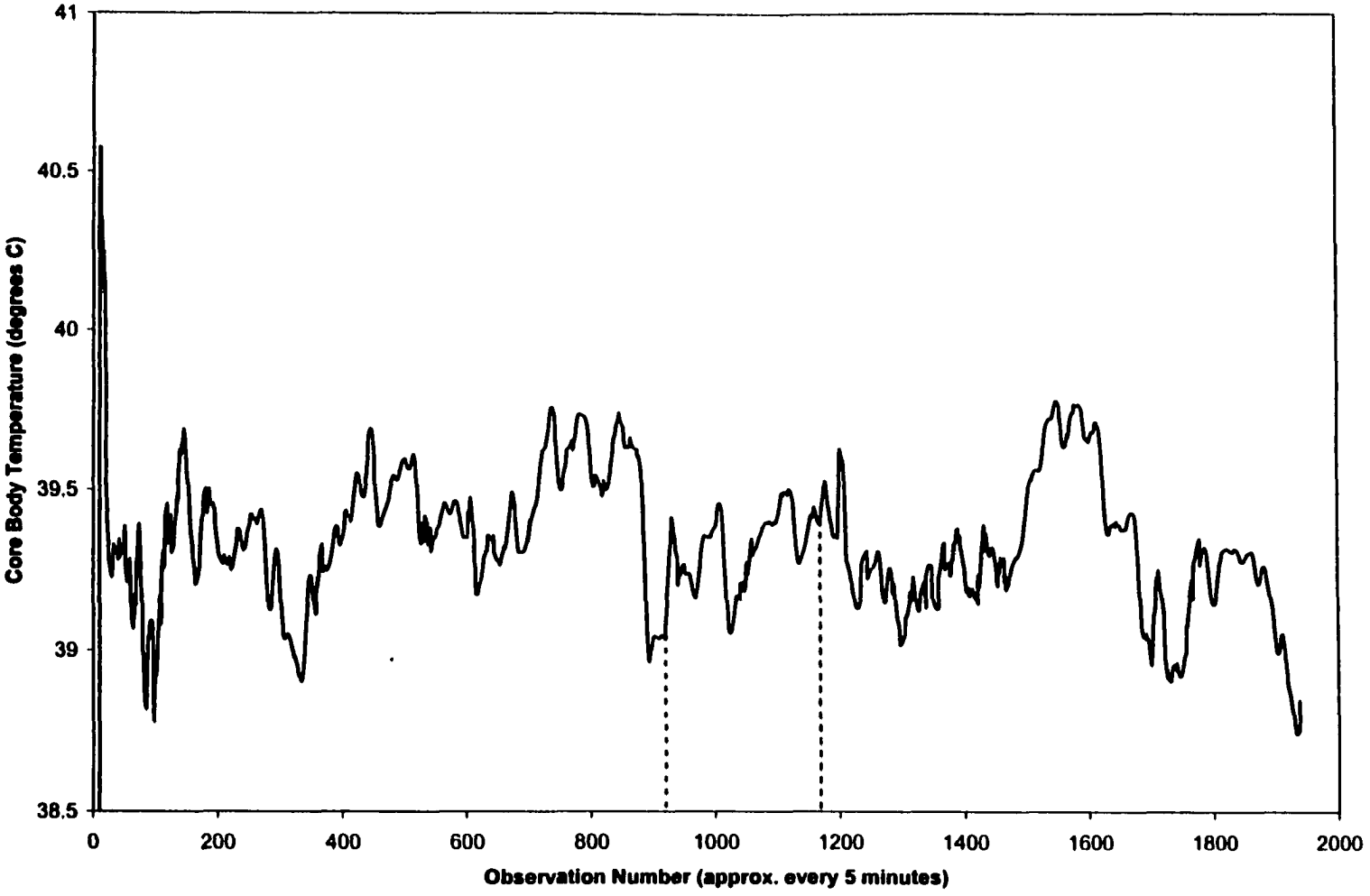

Figure 22. Animal No. 43; non-challenged

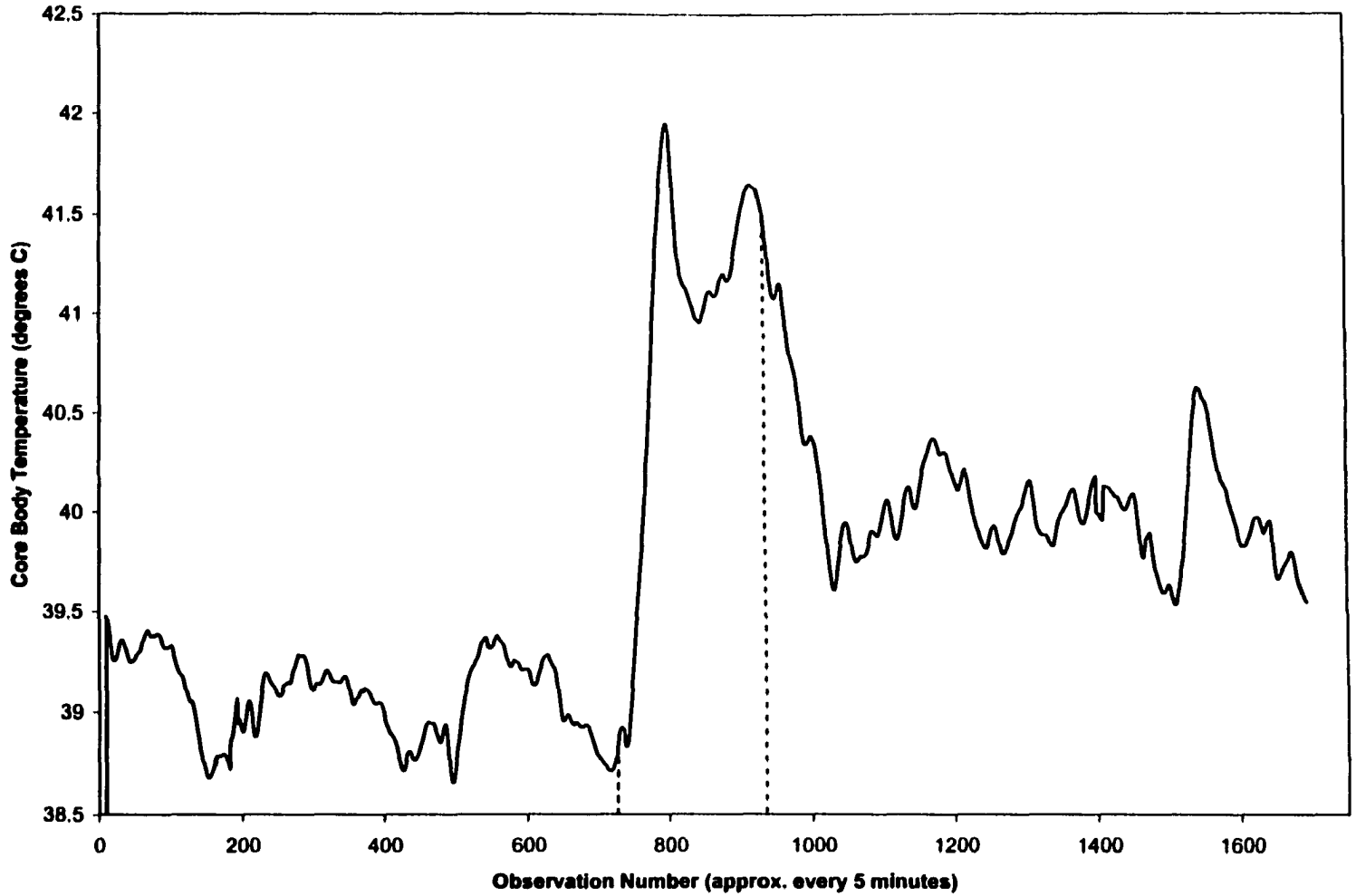

Figure 23. Animal No. 45; treated with tilmicosin

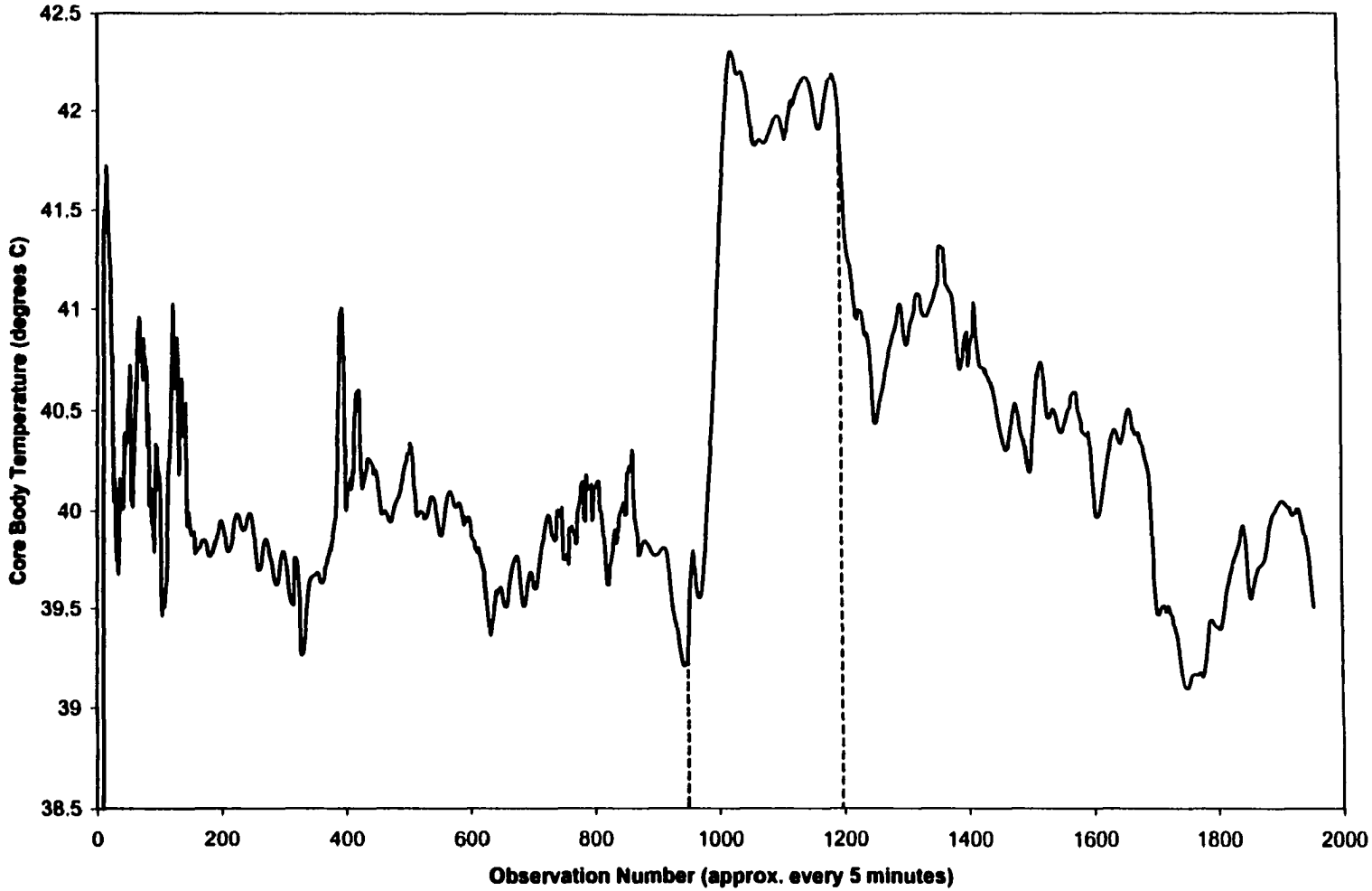

Figure 24. Animal No. 47; treated with saline

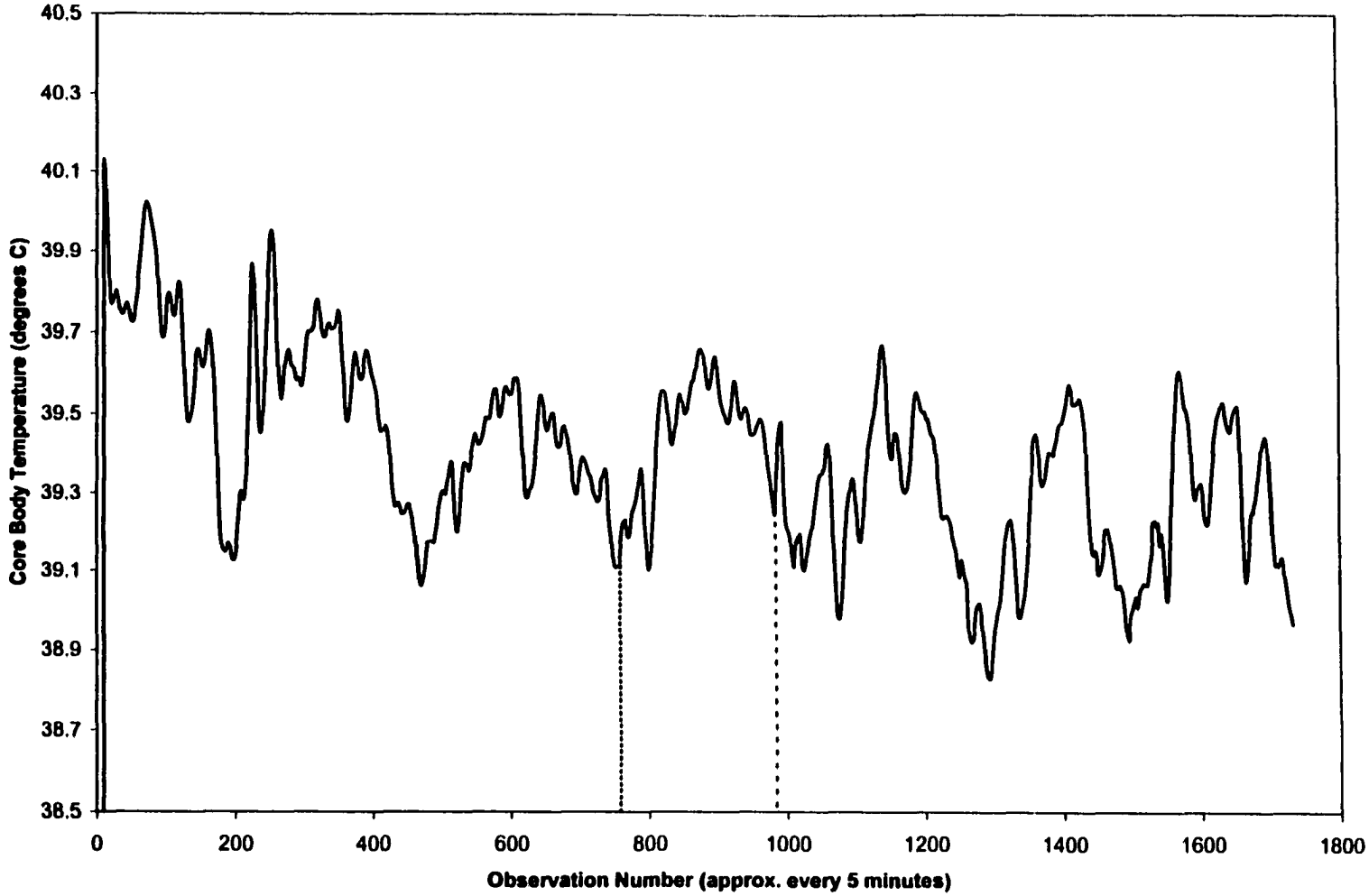

Figure 25. Animal No. 48; non-challenged

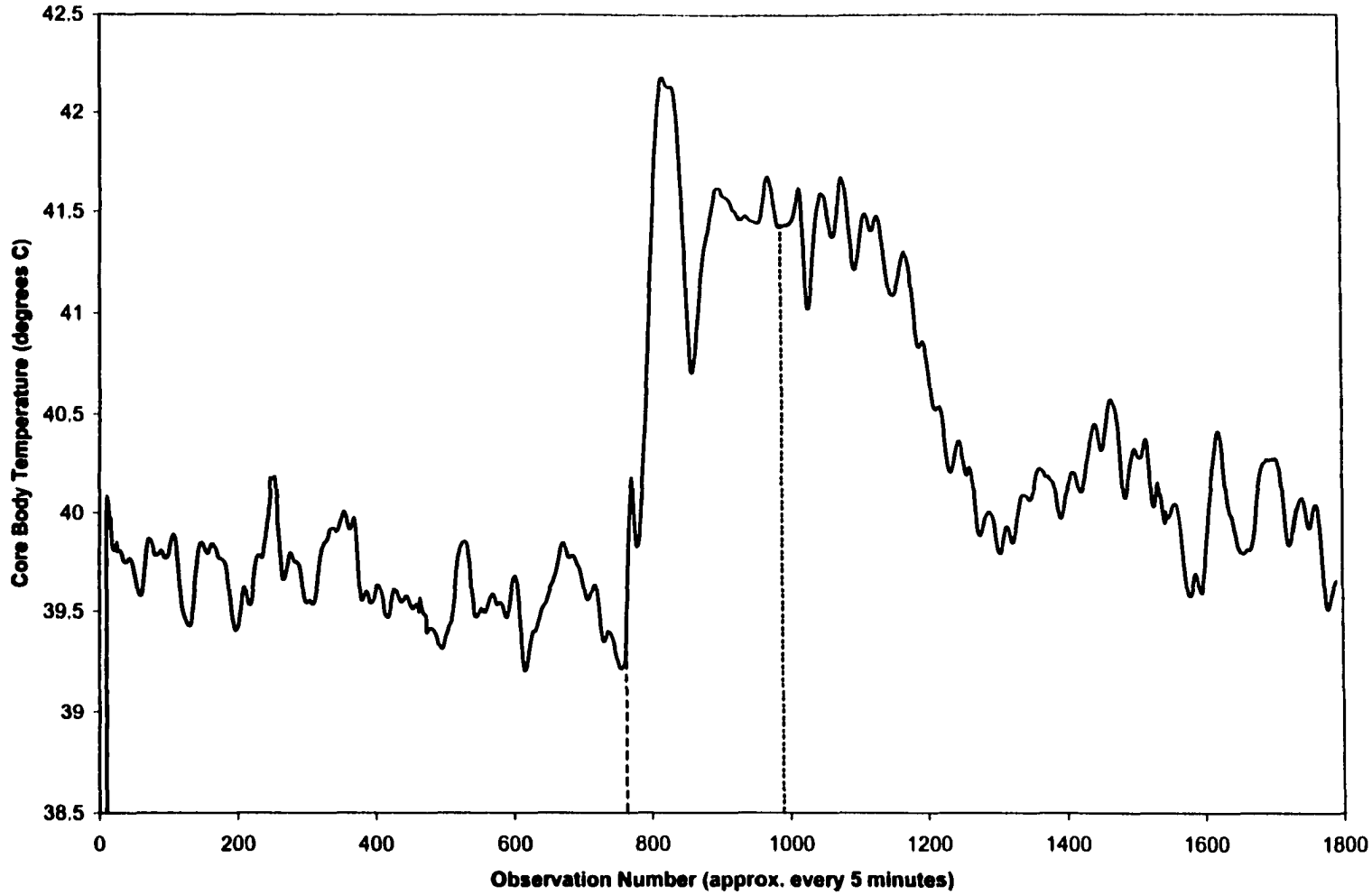

Figure 26. Animal No. 49; treated with saline

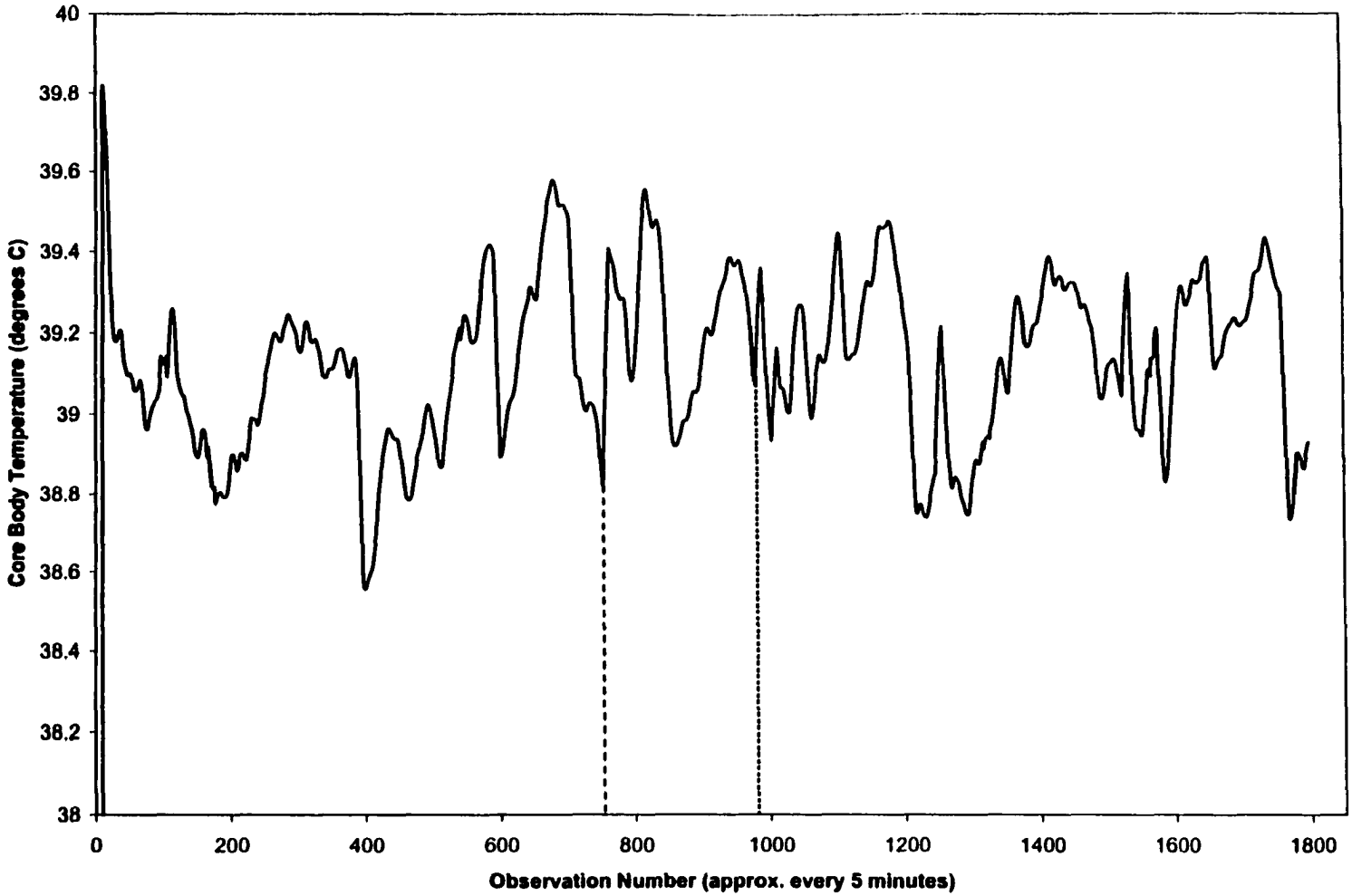

Figure 27. Animal No. 52; non-challenged

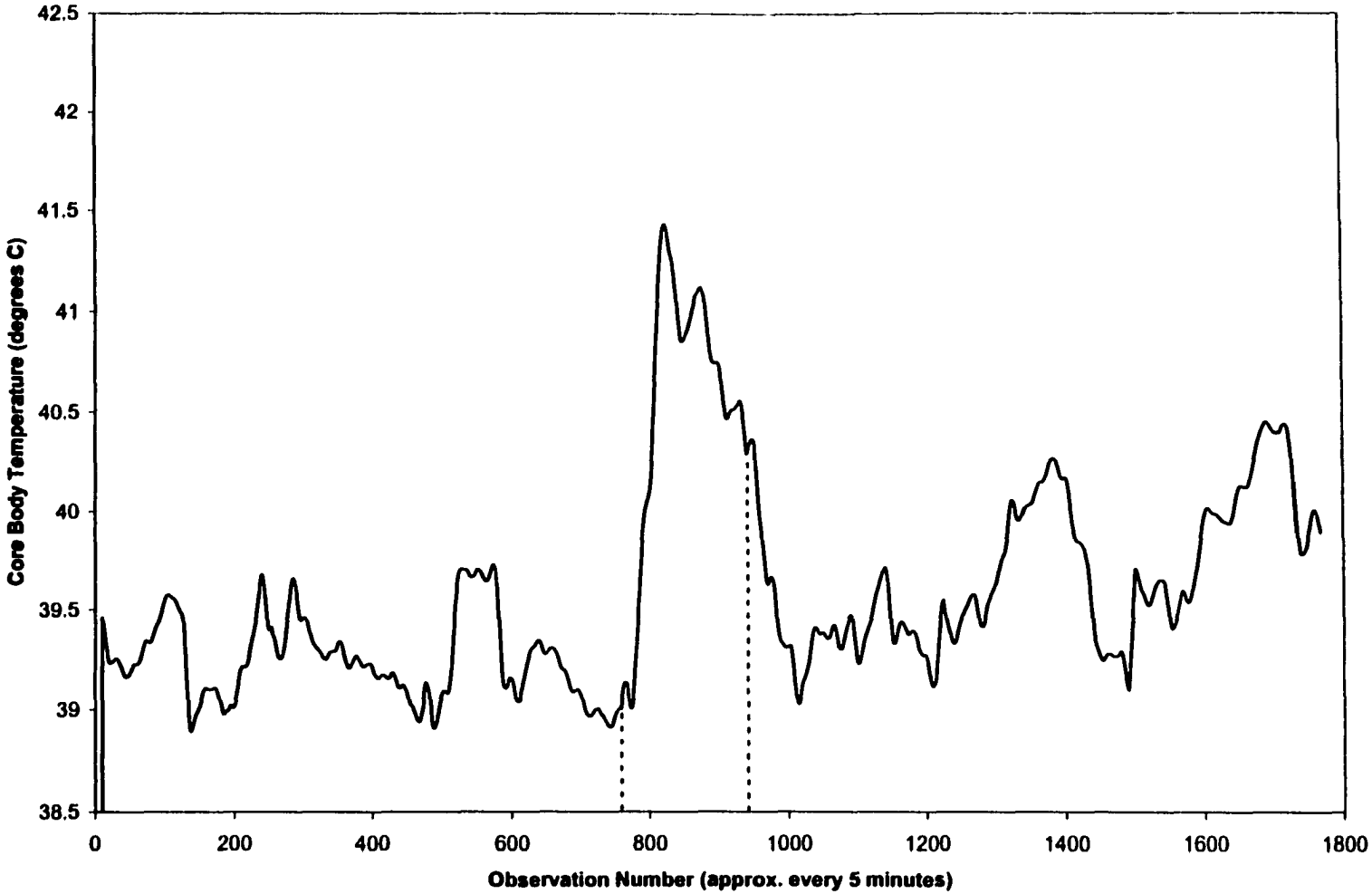

Figure 28. Animal No. 53; treated with danofloxacin

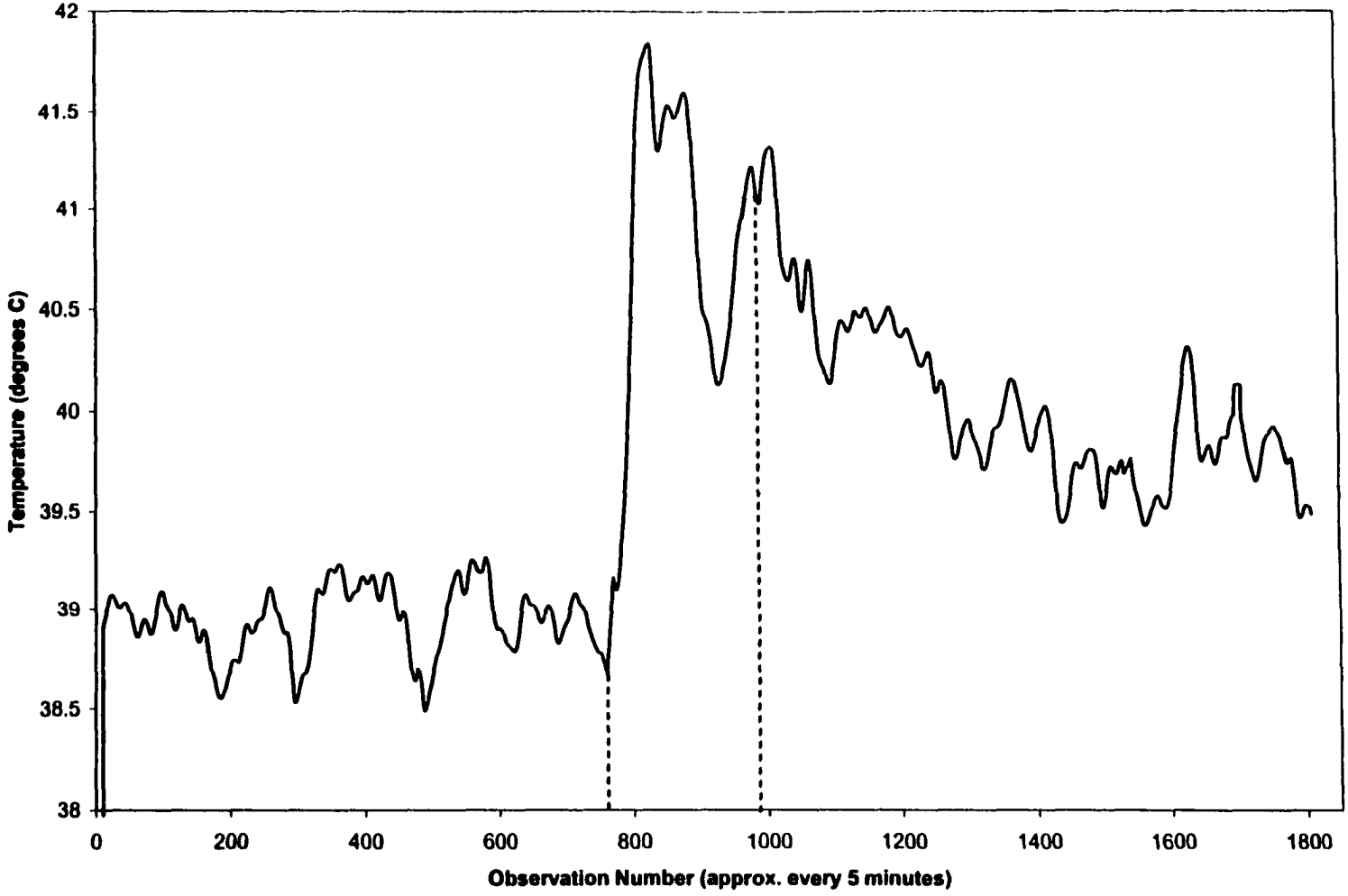

Figure 29. Animal No. 54; treated with tilmicosin

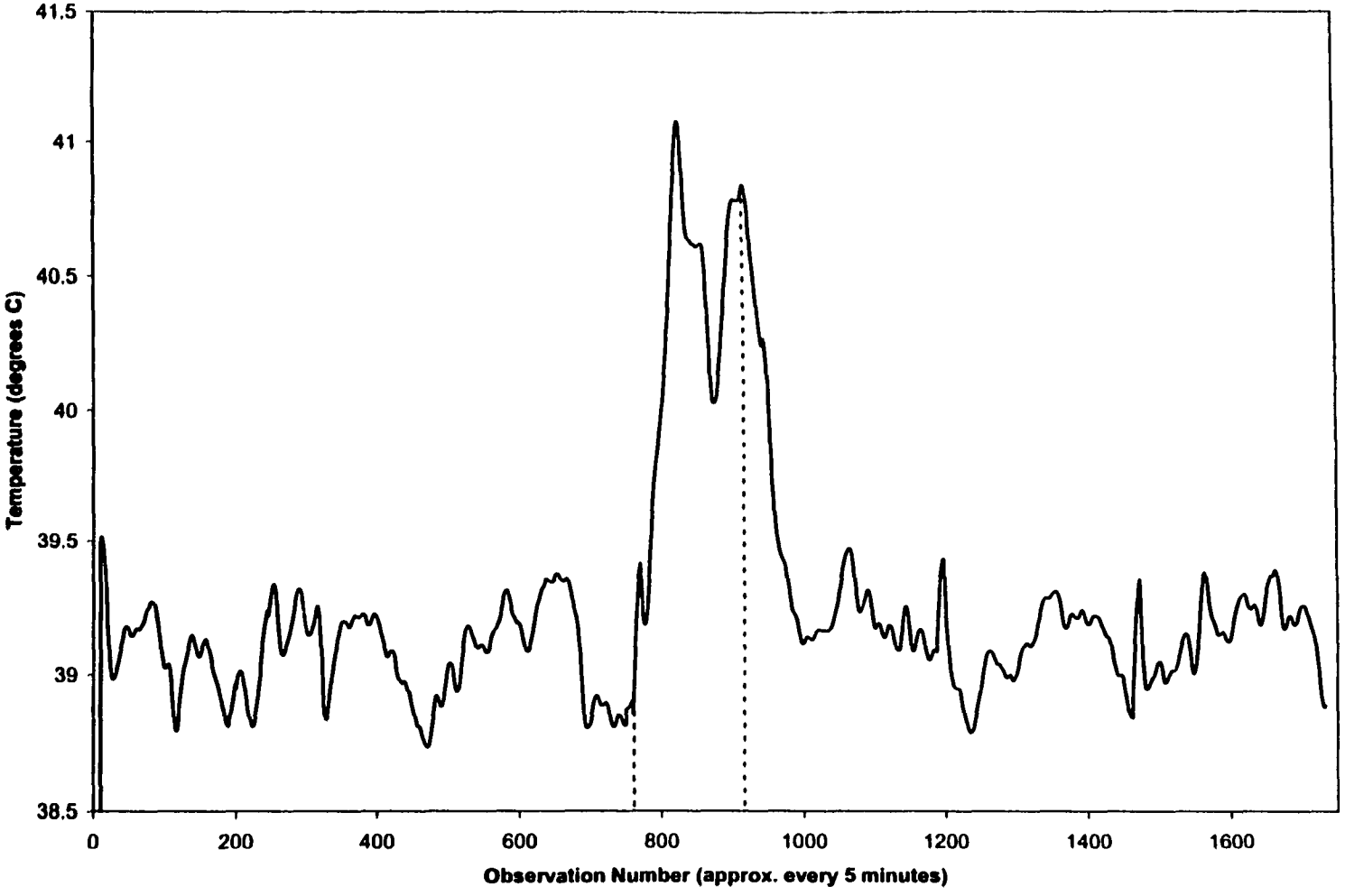

Figure 30. Animal No. 55; treated with tilmicosin

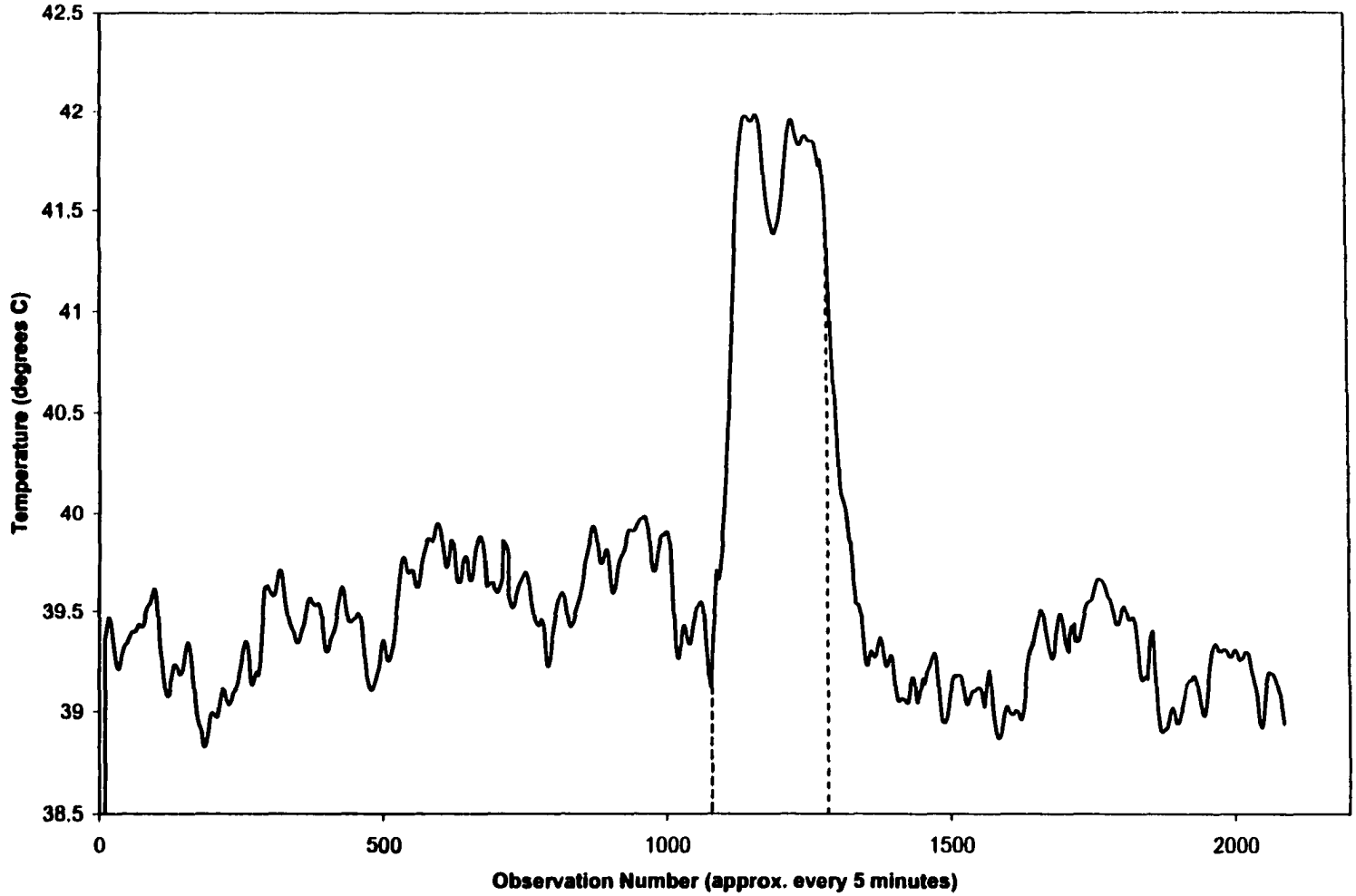

Figure 31. Animal No. 57; treated with danofloxacin

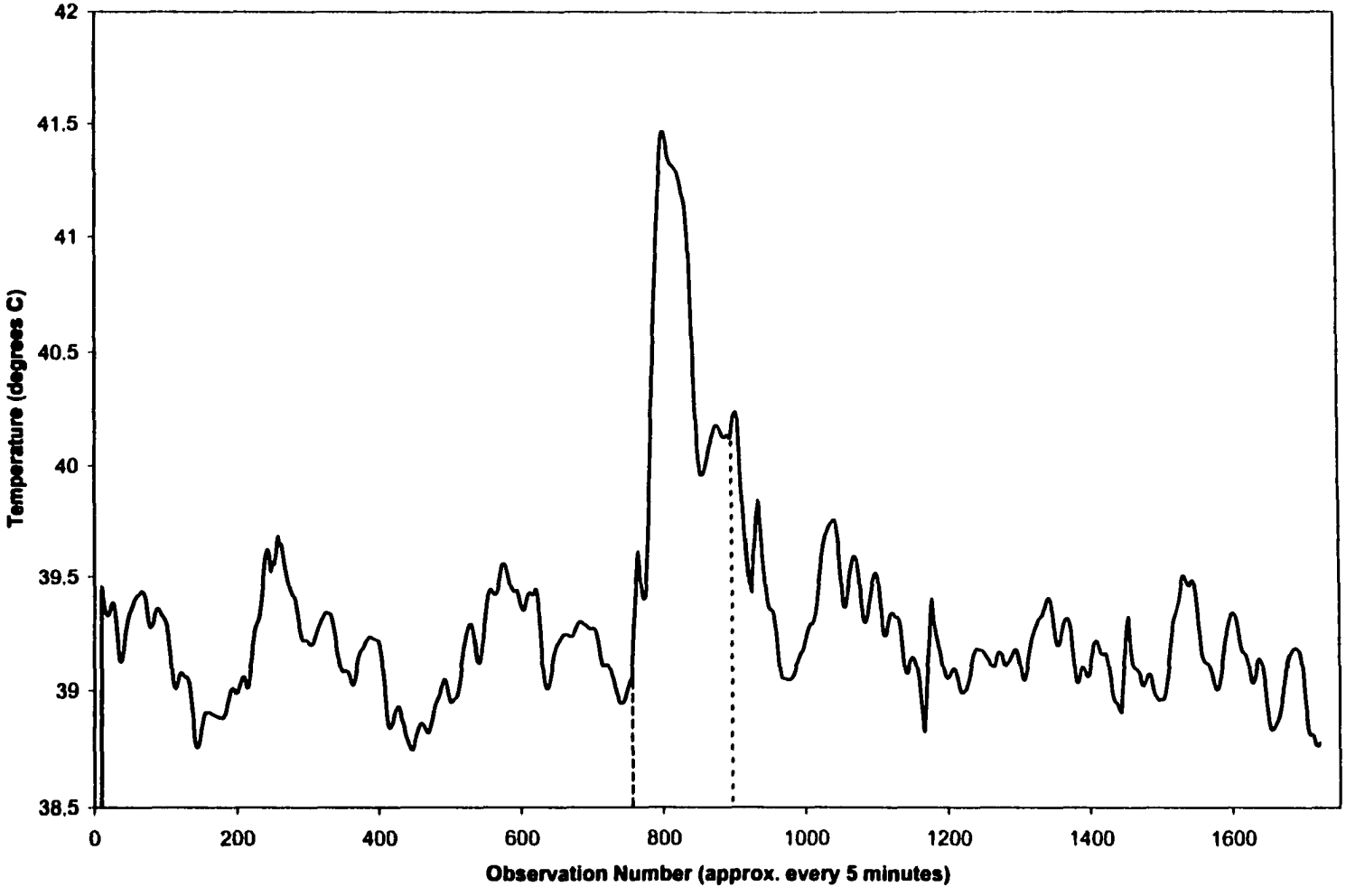

Figure 32. Animal No. 59; treated with tilmicosin