Treatment of Experimentally Induced Pneumonic Pasteurellosis of Young Calves with Tilmicosin

D.W. Morck, J.K. Merrill, M.S. Gard, M.E. Olson, and P.N. Nation

ABSTRACT

Twenty four (24) healthy male Holstein calves (< 70 kg) were each experimentally infected by intrabronchial inoculation of 4.0×10^9 viable cells of *Pasteurella haemolytica*-A1 (B122) at Time = 0 h. At 1 h following inoculation animals received either: 1) Sham treatment with sterile 0.85% saline SC ($n = 12$); or 2) a single injection of 10 mg tilmicosin per kg body weight ($n = 12$). Calves that were non-infected and tilmicosin-treated were also included for determining tilmicosin concentrations in serum and lung tissue at 1, 2, 4, 6, 8, 24, 48, and 72 h ($n = 3$ per time). In the infected calves, response to therapy was monitored clinically. Serum samples were collected for determination of tilmicosin concentrations using HPLC. Any animal becoming seriously ill was humanely killed. Complete necropsy examinations were performed on all animals and included gross pathologic changes, bacteriologic analysis, histopathology, and determination of pulmonary concentrations of tilmicosin. Tilmicosin treated animals responded significantly better to therapy than saline-treated control calves. Clinical assessment of calves during the study indicated that tilmicosin-treated calves had significantly improved by T = 8 h compared to saline-treated animals ($P < 0.05$). At necropsy tilmicosin-treated calves had significantly less severe gross and histological lesions ($P < 0.05$) of the pulmonary tissue. Of the 12 saline-treated calves, 92% (11/12) had *Pasteurella haemolytica*-A1 in lung tissue, while of the tilmicosin-treated calves 0% (0/12) cultured positive for *P. haemolytica*. Mean (± standard error) serum tilmicosin concentrations in infected calves peaked at 1 h post-injection (1.10 ± 0.06 µg/mL) and rapidly decreased to 0.20 ± 0.03 µg/mL, well below the MIC of 0.50 µg/mL for *P. haemolytica*-A1 (B122), by 12 h. These serum concentrations were very similar to serum concentrations of tilmicosin in non-infected tilmicosin-treated calves. Lung tissue concentrations of the antibiotic were comparatively high, even at 72 h post-infection (6.50 ± 0.75 ppm). Lung tissue concentrations at 72 h were significantly higher in experimentally infected calves than in non-infected tilmicosin-treated animals ($P < 0.05$). These data demonstrate that tilmicosin was effective in treating experimentally-induced pneumonic pasteurellosis as determined by alleviation of clinical signs, pathological findings at post mortem, and presence of viable bacteria from the lung. Concentrations substantially above MIC for *P. haemolytica* were present in lung tissue even at 72 h following a single subcutaneous injection of 10 mg tilmicosin per kg body weight.

RÉSUMÉ

Vingt-quatre veaux mâles en santé de race Holstein pesant moins de 70 kg ont été infectés expérimentalement par inoculation intrabronchique à l'aide de $4,0 \times 10^9$ cellules viables de *Pasteurella haemolytica* A1 (B122) au temps T = 0. Une heure suivant cette inoculation, les animaux ont reçu, soit une injection sous-cutanée de saline stérile ($n = 12$) ou une injection de tilmicosin à raison de 10 mg/kg de poids corporel ($n = 12$). Un groupe de veaux non-infectés et traités au tilmicosin a également été inclus afin de procéder à la détermination des concentrations sériques et au niveau du tissu pulmonaire de tilmicosin après 1, 2, 4, 6, 8, 24, 48 et 72 heures ($n = 3$ pour chacun des temps). La réponse au traitement chez les veaux infectés était suivie par des examens cliniques. Des échantillons de sérum étaient prélevés pour en déterminer par chromatographie, les concentrations sériques de tilmicosin. Les animaux sévèrement touchés étaient euthanasiés de façon humanitaire. Une nécropsie complète a été effectuée sur tous les animaux et comprenait une évaluation des changements pathologiques macroscopiques et microscopiques, une analyse bactériologique et la détermination de la concentration pulmonaire de tilmicosin. Les animaux traités au tilmicosin ont mieux répondu au traitement que les animaux recevant de la saline. L'évaluation clinique des veaux durant cette étude a démontré que les animaux traités au tilmicosin présentaient une amélioration significative ($P < 0,05$) par rapport aux animaux recevant la saline dès T = 8 heures. Lors de la nécropsie, il a été observé que les veaux traités au tilmicosin avaient des lésions pulmonaires macroscopiques et microscopiques significativement moins sévères ($P < 0,05$). La souche de *P. haemolytica* A-1 a été ré-isolée à partir des poumons de 11 des 12 veaux traités avec de la saline alors qu'elle n'a

Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4 (Morck, Gard); Provel Division, Eli Lilly Canada, Calgary, Alberta (Merrill); Department of Microbiology & Infectious Diseases, University of Calgary, Calgary, Alberta, (Olson); Faculty of Medicine, University of Alberta, Edmonton, Alberta (Nation).

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été isolée chez aucun des 12 veaux traités au tilmicosin. La concentration sérique de tilmicosin (moyenne ± erreur standard) chez les veaux infectés atteignait un pic 1 h après l'injection (1,10 ± 0,06 μg/mL) et diminuait rapidement pour atteindre une concentration de 0,20 ± 0,03 μg/mL 12 h post-injection, bien en deçà de la CMI de 0,50 μg/mL de la souche *P. haemolytica* A-1 utilisée. Ces concentrations sériques étaient très similaires aux concentrations sériques de tilmicosin déterminées chez les veaux du groupe traité mais non infecté. Les concentrations tissulaires au niveau du poumon étaient élevées, et ce même 72 h post-infection (6,50 ± 0,75 ppm). Les concentrations au niveau du tissu pulmonaire à 72 h étaient significativement plus élevées ($P < 0,05$) chez les veaux infectés expérimentalement que chez les veaux traités mais non infectés. Ces données démontrent que le tilmicosin est efficace pour le traitement de pasteurellose induite expérimentalement tel que le démontre la diminution des signes cliniques et des changements pathologiques ainsi que l'absence de bactéries viables dans le poumon. Des concentrations nettement supérieures à la CMI de *P. haemolytica* étaient retrouvées dans le tissu pulmonaire et ce même 72 h après seulement une injection sous-cutanée de tilmicosin à un dosage de 10 mg/kg.

(Traduit par docteur Serge Messier)

INTRODUCTION

Respiratory diseases are the most frequently treated infections in Western Canadian feedlot cattle (1). Treatment rates as high as 50% are not uncommon in calves placed in feedlots in the autumn. Tilmicosin has been used successfully as a prophylactic for infection in feedlot animals (2) and is also an effective therapy (3,4) for undifferentiated bovine respiratory disease. The most frequent bacterial pathogen encountered in these infections is *Pasteurella haemolytica*-A1 (5). Young calves are also susceptible to infection with *P. haemolytica* as a secondary invader within the enzootic pneumonia complex (1). Data demonstrating the effectiveness of tilmicosin in young calves (< 70 kg) are not readily available. This investigation was undertaken to examine the efficacy of tilmicosin for treating experimental pasteurellosis in young Holstein calves (< 70 kg) and to monitor the concentrations of this drug in serum and lung tissue during the infection as well as in normal, healthy, non-infected calves.

MATERIALS AND METHODS

EXPERIMENTAL INFECTION

Experimental Animals — Twenty-four (24) healthy, Holstein calves were used for this part of the study. Animals were obtained from 3 sources and were housed indoors on dirt floor pens. All 24 animals were co-mingled throughout the study. Calves were less than 70 kg body weight (mean = 43.4 kg, median = 42.5 kg, range = 32.0 to 70.0 kg) and were uniquely identified by ear tags. They were fed quality milk replacer (UFA Co-op, Airdrie, Alberta) and non-medicated creep feed (United Feeds, Olds, Alberta) ad libitum with free access to clean drinking water. Animal care and experimental procedures were conducted according to the standards of the Canadian Council on Animal Care (6). The Life & Environmental Sciences Animal Care Committee of the University of Calgary, Alberta reviewed and approved of the objectives and procedures of this study.

Growth of Bacteria and Experimental Infection — Pasteurella haemolytica-A1 (strain B122) was grown aerobically on blood agar plates and a single colony was inoculated into brain-heart infusion (BHI) broth. After 14 h incubation at 37°C, a 0.1% vol/vol inoculation was performed into fresh BHI broth and the culture was grown to mid-logarithmic phase at 37°C without agitation. Bacteria were harvested by centrifugation (10 min at 3000 × *g*) washed in sterile phosphate buffered saline (pH 7.2) and suspended at a concentration of 4.0×10^8 cfu/mL. Bacteria used for the infection were enumerated initially by McFarland nephelometry. A standardized solution was diluted and representative dilutions spread on blood agar plates to determine the precise number of viable *P. haemolytica* delivered to each calf. Bacterial suspensions were held at 4°C until inoculated into the calves. Bacterial numbers were verified by serial dilution of the inoculum and spread plating on blood agar plates. Calves were experimentally infected at Time = 0. Animals were physically restrained in lateral recumbency and local anesthesia (2% Lidocaine, MTC Pharmaceuticals, Cambridge, Ontario) applied in the skin and subcutaneous tissues of the medial ventral tracheal area. A stab incision was made with a scalpel blade and a 2 × 14″ gauge needle was carefully inserted between 2 cartilaginous rings of the trachea. A sterile catheter made of polyethylene tubing was inserted through the needle into the tracheal lumen to approximately the level of the primary bronchi. A syringe containing 10 mL of inoculum was used to inject viable bacterial cells into the lower respiratory tract (total inoculum 4.0×10^9 cfu) followed by 2 mL of sterile saline to displace any remaining bacterial suspension. The catheter and needle were removed and the incision was sealed with tissue adhesive (Vet-Bond, 3M, St Paul, Minnesota, USA). A new catheter system and needle was used for each new calf.

Design and Animal Allotment — A randomized block design consisting of 2 animals per block was used. Factors considered in blocking were weight and source. Animals were randomly assigned to 1 of the following treatments: 1) negative control — 0.85% sterile saline at 0.03 mL per kg body weight; or 2) 10 mg tilmicosin per kg body weight (Provel Animal Health, Guelph, Ontario). Medications were administered by single subcutaneous injection in the neck 1 h following the intrabronchial inoculation with *P. haemolytica*.

Clinical Measurements — Rectal temperatures were taken from calves at −1, 0, 1, 2, 3, 4, 8, 12, 16, 20, 24, 36, 48, 60, and 72 h during the experimental trial. Clinical scores were determined blindly for each animal at the same times as rectal temperature measurements using the following subjective system:

1) General Condition and Alertness: normal = 0; inactive with group = 1; lethargic (head or ears drooping and dull eyes) = 2; downer = 3.

2) Respiratory Condition: normal = 0; tachypnea (> 30/min) = 1; mild

dyspnea = 2; severe forced expiration = 3.

3) Appetite and Fill: normal = 0; anorexic = 1; very gaunt = 2; gaunt and dehydrated (skin turgor) = 3.

4) Nasal Discharge: none = 0; serous = 1; mucopurulent = 2; purulent = 3.

5) Ocular Discharge: none = 0; serous = 1; mucopurulent = 2; purulent = 3.

6) Feces: normal = 0; diarrhea = 1.

7) Inducible Cough: none = 0; mild = 1; severe = 2.

Whole blood samples were collected for serum at 0, 1, 2, 3, 4, 8, 12, 24, 48, and 72 h. Blood samples were allowed to clot and centrifuged at 1000 × *g* (20 min) to separate the serum. Sera were held frozen at −85°C until analyzed for tilmicosin concentration. Lung tissues were obtained from animals at 72 h and assayed for tilmicosin concentrations by reverse phase High Performance Liquid Chromatography (HPLC) (7).

Postmortem Examinations — Any animal that became severely ill, and all remaining animals at 72 h following experimental inoculation with *P. haemolytica*, were heavily sedated with intravenous sodium pentobarbital and euthanized by exsanguination. Postmortem examinations were conducted concentrating on the respiratory tract. The prosector was blinded to treatment groupings. Gross pathologic findings were scored as follows: no consolidation = 0; slight consolidation (< 5% involved) = 1; moderate consolidation (6%–20% involved) = 2; severe consolidation (> 20% involved) = 3.

Bacteriology — Swabs of affected lung tissue were taken at postmortem, placed in Amies transport medium containing activated charcoal, and immediately transported to the laboratory. Samples were plated on blood agar and incubated at 37°C under 5% CO_2 for 48 h. Predominant colony types were isolated in pure culture and identified by standard methodology (8). Minimum inhibitory concentrations (9) of the predominant isolates were determined for tilmicosin using agar dilution.

Histology and Histopathological Scoring — Tissue specimens of lesions (3 unique areas per calf) were obtained and fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The following system was used in which hemorrhage, consolidation, and inflammation were independently scored in each tissue section and scores totaled to obtain a pneumonia score. The scoring included:

1) Hemorrhage; Absent = 0; Focal hemorrhage less than or equal to 1/10 power field = 1; Multiple focal hemorrhages greater than 1/10 power field = 2; Diffuse or confluent hemorrhage occupying more than 50% of each 10 power field = 3.

2) Consolidation; Normal alveolar spaces = 0; Greater than or equal to 50% of alveoli open per 4 power field = 1; 30% to 50% of alveolar spaces open per 4 power field = 2; 0% to 29% of alveolar spaces open per 4 power field = 3.

3) Inflammation; No inflammation = 0; Mild non-fibrinous inflammation = 1; Acute fibrinous inflammation involving < 50% of each 4 power field or suppurative; alveolitis/bronchial pneumonia/subacute fibrinous change involving > 50% of each 4 power field = 2; Acute fibrinous pneumonia with necrosis involving ≥ 50% of each 4 power field = 3.

In order to be classified as fibrinous pneumonia all of the following had to be present: edema, fibrin exudation into the alveoli and/or interstitial connective tissue, neutrophils in alveoli, macrophages in alveoli, and oat cells in alveoli.

Statistical Analysis — Discrete data were analyzed using chi-square ($P < 0.05$). Continuous non-parametric data were analyzed using Mann-Whitney ($P < 0.05$) or Kruskal-Wallis and Dunn's multiple comparisons ($P < 0.05$).

PHARMACODYNAMIC STUDY

Infected Calves — Tilmicosin-treated calves from the experimental infection, as described above, were included as part of a pharmacodynamic study. These animals ($n = 12$) were sequentially sampled for serum at 0, 1, 2, 3, 4, 8, 12, 24, 48, and 72 h. Lung samples were collected from the calves at the time of necropsy examination, as described above.

Non-infected Calves — Twenty-four (24) clinically normal, healthy, Holstein calves were used in this part of the study. All calves were male and less than 70 kg in body weight (mean = 50.6 ± 1.55 kg; median = 51.5 kg; range 34.5 to 61.5 kg). Two additional calves of similar weight were untreated with the antibiotic and served as controls for HPLC assays. Each calf was provided with unique identification using ear tags and animals were comingled in a single housing unit. A randomized block design was used for the study involving pharmacodynamics in normal, healthy calves. Each block consisted of 8 calves and the animals were blocked according to weight and source. All 24 calves were given a single injection of tilmicosin at 10 mg/kg body weight. Three animals were euthanized at each of the following times post-injection: 1, 2, 4, 6, 8, 24, 48, and 72 h. Whole blood for serum and tissue samples of exsanguinated lung were collected from calves. Assignment of animals from within blocks to sampling times was conducted using a random numbers table. The individual animal was the experimental unit. The 2 control animals (no antibiotic) were sampled as above and were used only for external controls of extractions and HPLC analyses. Serum and lung tissues from normal, healthy calves were extracted with chloroform and assayed for concentrations of tilmicosin by reverse-phase HPLC, according to previously established methodology (7).

Statistical Analysis — Continuous non-parametric data were analyzed using Mann-Whitney ($P < 0.05$) or Kruskal-Wallis and Dunn's multiple comparisons ($P < 0.05$).

RESULTS

EXPERIMENTAL INFECTION

Inoculum and Experimental Infection — The inoculum estimate was confirmed as containing 4.0×10^8 cfu/mL by direct viable bacterial cell counts. Infection was induced successfully in all 24 calves subjected to intrabronchial instillation of *Pasteurella haemolytica*. Within 1 h of inoculation, the animals in both groups developed elevated rectal temperatures and began demonstrating clinical signs of respiratory tract infection including lethargy, dullness, abnormal respiratory sounds upon auscultation, and altered rate and depth of breathing pattern.

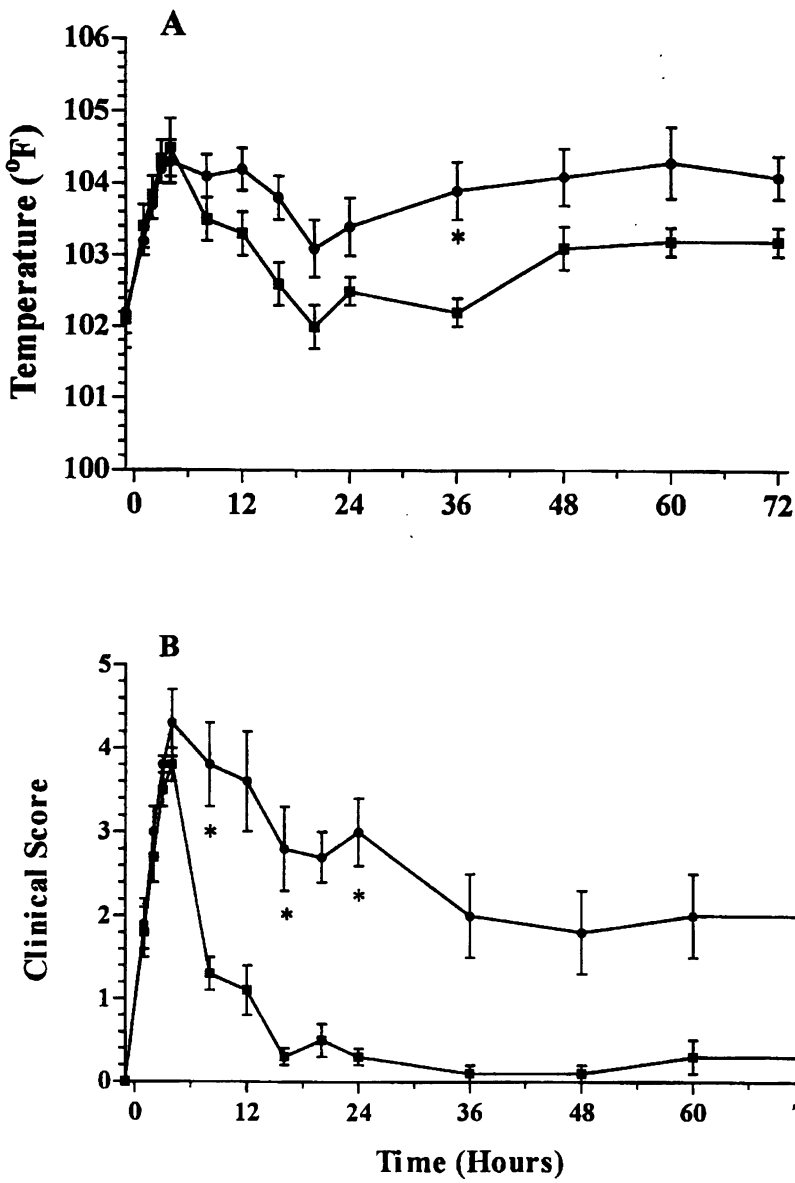

Figure 1. (A) A graph of the rectal temperatures of saline-treated control calves (●) and tilmicosin-treated calves (■) following experimental infection with *P. haemolytica*. Values are means ± standard errors. Statistical significance ($P < 0.05$) is represented with *. [Rectal temperature (°F) vs. time (Hours)]. (B) Graph of the clinical scores of saline treated control calves (●) and tilmicosin treated calves (■) following experimental infection with *P. haemolytica*. Values are means ± standard errors. Statistical significance ($P < 0.05$) is represented with *. [Clinical score vs. time (Hours)].

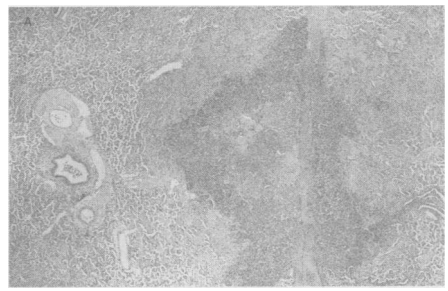

Figure 2A. A representative photomicrograph of pulmonary tissue from saline treated control animals. This H & E stained section depicts extensive hemorrhage, necrosis, consolidation, fibrin deposition, and infiltration of inflammatory leukocytes (primarily neutrophils). (40×).

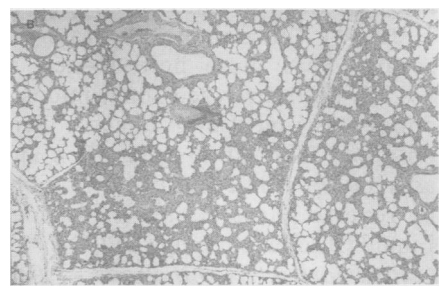

Figure 2B. A representative photomicrograph of pulmonary tissue from tilmicosin treated animals. This H & E stained section depicts a reasonably normal lung, however, slight interstitial reaction is present. (40X).

Clinical Measurements — The rectal temperatures of saline-treated and tilmicosin-treated calves are illustrated in Figure 1A. Rectal temperatures were significantly elevated ($P < 0.05$) in calves from both groups within 3 h when compared to pre-inoculation rectal temperatures. At each time point following experimental infection saline-treated animals demonstrated temperatures higher than those of tilmicosin-treated calves, but this increase was only significant at 36 h ($P < 0.05$). Clinical scores for saline-treated control and tilmicosin-treated animals are shown in Figure 1B. In both saline-treated and tilmicosin-treated animals the clinical scores became significantly elevated within 2 h of experimental infection when compared to pre-inoculation assessments ($P < 0.05$). In saline-treated calves the clinical scores remained significantly elevated for the duration of the experiment; however, by 8 h in tilmicosin-treated animals scores were no different than pre-exposure scores ($P > 0.05$). When comparing saline-treated animals and tilmicosin-treated animals the clinical scores were significantly lower ($P < 0.05$) in the tilmicosin group at 8, 16, and 24 h, respectively.

Postmortem Examinations and Bacteriology — Four (4) animals in the saline control group became severely ill and euthanized prior to the end of the study at 72 h following infection. This was significantly more than those requiring euthanasia in the tilmicosin treated group (0/12) ($P < 0.05$). Grossly evident consolidation of the lung was significantly more severe in saline-treated (33.5 ± 6.5%) than in tilmicosin-treated (7.3 ± 3.2%) calves ($P < 0.05$). *Pasteurella haemolytica* was isolated from the lungs of 92% (11/12) of the saline controls at the time of necropsy, but not from any (0/12) of the lungs of tilmicosin-treated calves at 72 h following experimental infection. The isolates of *P. haemolytica* obtained from the saline-treated animals had MICs for tilmicosin of 0.5 μg/mL and all possessed identical antibiograms (data not shown) to the

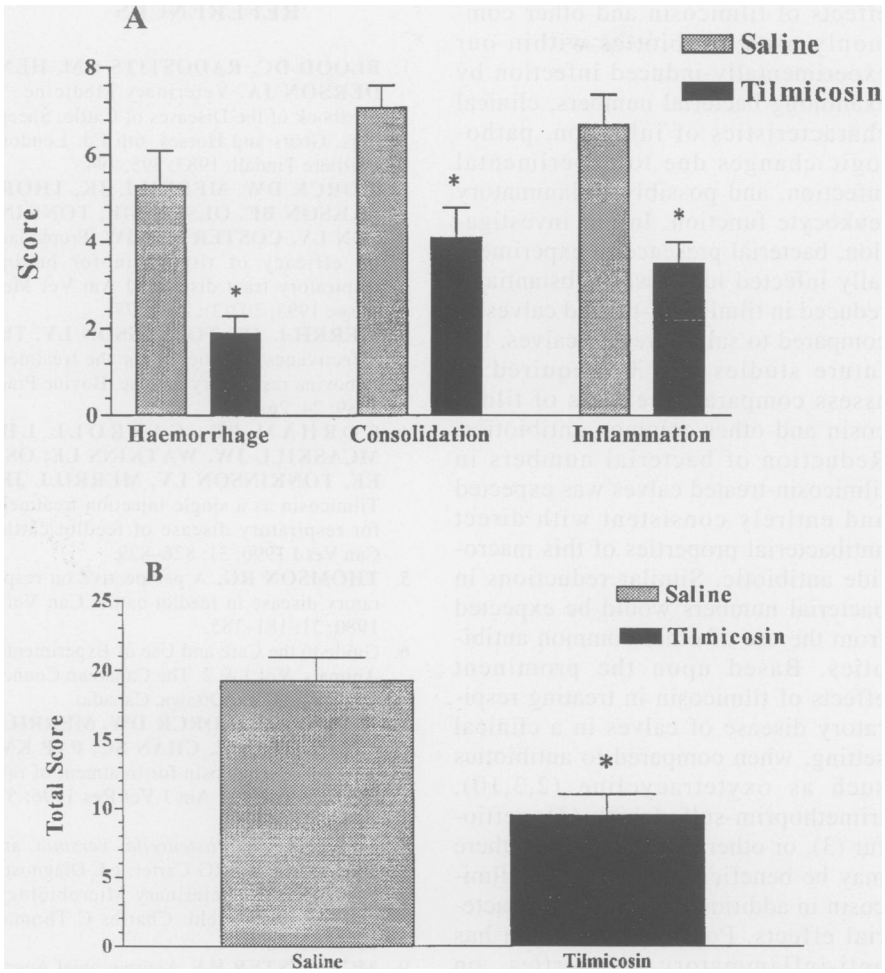

Figure 3. (A) A histogram representing the histopathological scores for hemorrhage, consolidation and inflammation in saline treated calves and tilmicosin treated calves. Statistical significance ($P < 0.05$) is represented with *. [Respective score vs. experimental group]. (B) A histogram comparing the histopathological total pneumonia scores in saline treated calves and tilmicosin treated calves. Statistical significance ($P < 0.05$) is represented with *. [Total score vs. experimental group].

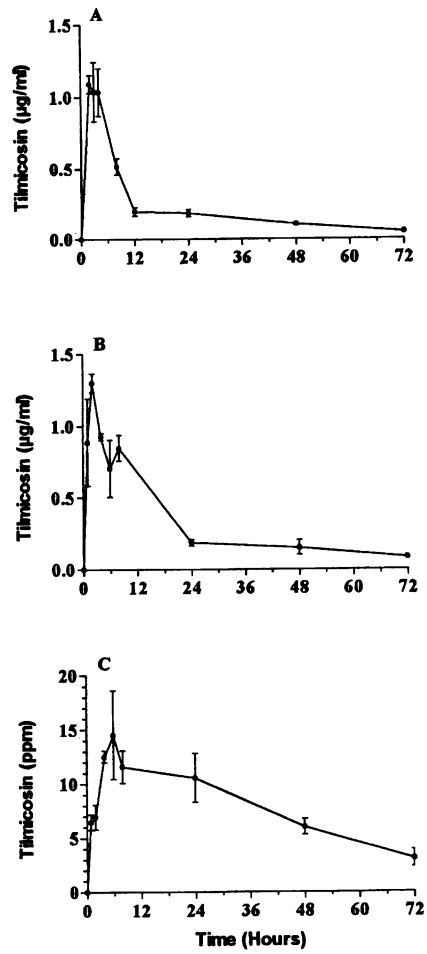

Figure 4. (A) Graph showing the concentrations of tilmicosin in serum of experimentally infected calves ($n = 12$). [Tilmicosin (µg/mL) vs. time following experimental infection (Hours)]. (B) Graph showing the concentrations of tilmicosin in serum of non-infected calves ($n = 3$ per data point). [Tilmicosin (µg/mL) vs. time following tilmicosin administration (Hours)]. (C) Graph showing the concentrations of tilmicosin in lung of non-infected calves ($n = 3$ per data point). [Tilmicosin (ppm) vs. time following tilmicosin administration (Hours)].

inoculum strain (B122) as measured by a Kirby Bauer technique using 11 separate antibiotics. These data indicate the recovered strain was the strain experimentally placed in the lungs of these calves.

Histology and Histopathological Scoring — Photographs of representative histopathologic changes in the 2 experimental groups are presented in Figures 2A and 2B. Saline-treated animals (2A) had extensive areas of coagulation necrosis of pulmonary tissue, hemorrhage, consolidation, fibrin deposition, and infiltration with neutrophilic granulocytes. While not completely normal, the tilmicosin-treated calves had fewer and less severe microscopically detectable lesions of the lung (2B). When these lesions were scored blindly and independently, tilmicosin-treated calves had significantly less severe hemorrhage, consolidation and inflammation ($P < 0.05$) (Figure 3A) than controls. Total pneumonia scores were significantly lower ($P < 0.05$) in tilmicosin-treated calves compared to saline-treated animals (Figure 3B).

PHARMACODYNAMIC STUDY

Infected Calves — Serum concentrations of tilmicosin in infected calves are illustrated in Figure 4A. Serum concentrations peak rapidly (within 1 h of administration) and very quickly decline over the next 8–12 h. Concentration of tilmicosin in lung tissue of infected calves at 72 h was 6.50 ± 0.75 ppm.

Non-infected Calves — Concentrations of tilmicosin in serum from non-infected calves (Figure 4B) were very similar to those in infected calves. The concentration of tilmicosin in lung tissue at 72 h in non-infected calves was 3.1 ± 0.81 ppm, and this was significantly lower ($P < 0.05$) than in infected tilmicosin-treated calves. Lung concentrations of tilmicosin in normal, healthy calves at several time points following administration of the antibiotic are graphed in Figure 4C. Comparatively high concentrations were rapidly achieved in lung tissue and they persisted until the end of the experiment at levels substantially above the MIC for this strain of *Pasteurella*.

DISCUSSION

Tilmicosin has been widely utilized as an antimicrobial agent for *Pasteurella* infections in feedlot cattle in North America (2–4) and in Europe (10,11). Previous reports have suggested that the pharmacological characteristics and antibacterial properties of this macrolide antibiotic (12) render it particularly useful for *Pasteurella haemolytica* infections of the lung. The present experimental data confirm the effectiveness of tilmicosin for the treatment of pasteurellosis in very young calves (< 70 kg). Of interest in this study were the positive effects of this antibiotic in a provocatively challenged model system of pasteurellosis of the lungs. The severe infection induced was profoundly modified by the single administration of tilmicosin. Presence of bacteria and neutrophils as well as tissue damage in infected pulmonary tissue were significantly reduced by the presence of this antibiotic.

Tilmicosin rapidly accumulates in lung tissue and significantly higher concentrations of the antibiotic are found in infected pulmonary tissue as compared to non-infected lungs. This may be a result of physical and chemical conditions associated with pulmonary inflammation or there may be a specific accumulation of the antibiotic associated with the cells infiltrating the septic lung.

Studies of experimentally-induced pneumonia comparing tilmicosin with other antibiotics commonly used for pasteurellosis have not been conducted. Gourlay et al (13) published a study examining bacteriologic, clinical, and pathologic benefits of tilmicosin in *Mycoplasma* and *Pasteurella* infected calves; however, the study was small, more extensive pharmacological data would have been useful, and specific assessment of histopathologic changes by blinded scoring was not performed. Our clinical data confirmed findings of these investigators (13), and we examined more precisely the tissue damage in a semiquantitative fashion. Most published evaluations of tilmicosin have been conducted in a purely clinical situation (2–4, 10, 11). Therefore, it would be interesting to comparatively assess effects of tilmicosin and other commonly used antibiotics within our experimentally-induced infection by examining bacterial numbers, clinical characteristics of infection, pathologic changes due to experimental infection, and possibly inflammatory leukocyte function. In our investigation, bacterial presence in experimentally infected lungs was substantially reduced in tilmicosin-treated calves as compared to saline-treated calves, but future studies will be required to assess comparative effects of tilmicosin and other common antibiotics. Reduction of bacterial numbers in tilmicosin-treated calves was expected and entirely consistent with direct antibacterial properties of this macrolide antibiotic. Similar reductions in bacterial numbers would be expected from the use of other common antibiotics. Based upon the prominent effects of tilmicosin in treating respiratory disease of calves in a clinical setting, when compared to antibiotics such as oxytetracycline (2,3,10), trimethoprim-sulfadoxine (3), ceftiofur (3), or other antibiotics (11), there may be beneficial properties of tilmicosin in addition to directly antibacterial effects. Possibly tilmicosin has anti-inflammatory properties on bovine leukocytes and endothelial cells. Additional investigations into the precise mechanisms involved in the clinical and pathological improvements seen with the use of tilmicosin will be required.

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