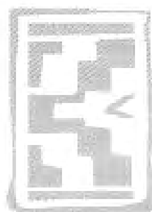

The clinical efficacy of enrofloxacin in the treatment of experimental bovine pneumonic pasteurellosis

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ABSTRACT

THOMPSON, P.N., VAN AMSTEL, S.R. & HENTON, M. 1998. The clinical efficacy of enrofloxacin in the treatment of experimental bovine pneumonic pasteurellosis. *Onderstepoort Journal of Veterinary Research*, 65:105–112

The clinical efficacy of enrofloxacin was tested in calves with experimentally induced pneumonic pasteurellosis. A strain of *Pasteurella haemolytica*, biotype A, serotype 1 (*P. haemolytica* A1), which had been isolated from an outbreak of pneumonic pasteurellosis in feedlot calves, was used to induce the disease in 24 eight-month-old calves. Each animal received, by intratracheal injection, 6 x 10^{11} colony forming units of *P. haemolytica* A1 in a four-hour log phase culture. Twelve similar animals were kept as non-infected controls (Negative Control group). Treatment of the infected animals commenced 40 h after infection and was as follows: 12 animals each received 2,5 mg/kg enrofloxacin subcutaneously and 12 animals each received 5 mℓ sterile saline intramuscularly (Positive Control group). All treatments were given once daily for three consecutive days. Clinical examinations were performed on all animals once daily, starting prior to infection and continuing until 12 d post-infection. The parameters evaluated were rectal temperature, habitus (attitude), ocular mucous membrane congestion and abnormal sounds on lung auscultation. On day14 post-infection, all animals were killed and their lung lesions (if any) estimated as the percentage involvement of each pair of lungs. The only statistically significant ($P \geq 0,05$) differences observed were between the Negative Control group and the Positive Control group. Noticeable differences were seen between the enrofloxacin-treated group and the Positive Control group, but they were not statistically significant ($P > 0,05$). The average lung lesion score (pneumonic lesions as a percentage of total lung volume) for the Positive Control group was 12,1% and that of the enrofloxacin-treated group, 8,4%. This difference was not statistically significant ($P > 0,05$).

Keywords: Bovine, enrofloxacin, *Pasteurella haemolytica*, pneumonic pasteurellosis

INTRODUCTION

Pneumonic pasteurellosis is an acute respiratory infection of cattle which is of major economic significance to the beef cattle industry in many countries, including the United States of America (Whiteley, Maheswaran, Weiss, Ames & Kannan 1992), Great Britain (Selman, Allan, Dalgleish, Gibbs, Shoo, Wiseman & Young 1986) and South Africa (Nesbit & Van Amstel 1994). It is generally accepted that the principal agent responsible for producing the characteristic fibrinous bronchopneumonia seen in this disease is *Pasteurella haemolytica*, biotype A, serotype 1 (*P. haemolytica* A1) (Whiteley *et al.* 1992). The natural disease condition is almost invariably associated with conditions of stress and/or viral respiratory infections (Yates 1982).

Several models have been described for the experimental induction of pneumonic pasteurellosis in calves. These include intranasal challenge with *P. haemolytica* preceded by aerosol challenge with either bovine herpesvirus-1 (BHV-1) (Jericho & Langford 1978; Stockdale, Langford & Darcel 1979; Cho &

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Accepted for publication 19 March 1998—Editor

Jericho 1986; Gifford, Potter & Babiuk 1988) or parainfluenza-3 (PI-3) virus (Cho & Jericho 1986). Preparations containing *P. haemolytica* alone have been used to induce the disease by various routes of administration, including transthoracic (Panciera & Corstvet 1984), intratracheal (Ames, Markham, Opuda-Asibo, Leininger & Maheswaran 1985), endobronchial (Vestweber, Klemm, Leipold & Johnson 1990), a combination of intranasal and intratracheal (Gibbs, Allan, Wiseman & Selman 1984; Gilmour, Gourlay, Gilmour, Donachie & Jones 1986), and intravenous (Farrington, Jackson, Bentley & Barnes 1987).

Many antibacterial and other drugs have been used in the treatment of pneumonic pasteurellosis (Burrows 1985; Burrows, Barto & Weeks 1986; Selman *et al.* 1986; Lekeux & Art 1988; Hansen, Campbell, Boyle, Stefanides, Whitsett & Williams 1993) and recently several newer antibiotics, including the fluoroquinolones, have been used (Giles, Grimshaw, Shanks & Smith 1991). The fluoroquinolones are bactericidal, inhibiting replication of bacteria by interfering with the action of DNA gyrase (Vancutsem, Babish & Schwark 1990). They have a high volume of distribution and reach higher levels in lung tissue than in serum (Scheer 1987; Mann & Frame 1992). They are active against a wide range of Gram negative and Gram positive aerobic bacteria, including *P. haemolytica*, as well as *Mycoplasma* spp. and *Chlamydia* sp. (Giles, Magonigle, Grimshaw, Tanner, Risk, Lynch & Rice 1991). At present, two fluoroquinolones, enrofloxacin and danofloxacin, are commercially available in South Africa for use in cattle. This trial was undertaken to evaluate the clinical efficacy of enrofloxacin, at label dosage, in the treatment of experimentally induced pneumonic pasteurellosis in cattle.

MATERIALS AND METHODS

Animals

Thirty-six six- to eight-month-old, recently weaned Charolais-cross calves were used. They were obtained directly from a commercial farm where they had been reared as a single group on pasture, had received no vaccinations and had not been exposed to any animals from other sources. There was no history of respiratory disease occurring on the farm of origin. On arrival at the trial facility, each animal was individually identified by means of a plastic ear tag, dewormed with a broad-spectrum anthelminthic ("Ivomec Injectable", Logos Agvet, Halfway House, South Africa), treated with a pour-on ectoparasiticide ("Pouracide-NF", Pfizer Laboratories SA, Sandton, South Africa), and vaccinated against anthrax ("Anthrax Spore Vaccine", Onderstepoort Biological Products, Onderstepoort, South Africa), botulism and quarter-evil ("Botulism/Blackquarter Combination Vaccine", Onderstepoort Biological Products, Onderstepoort, South Africa).

Each animal was randomly assigned to one of three experimental groups of 12 animals each. Group 1 was the Negative Control group (neither infected nor treated); group 2 the Positive Control group (infected but not treated) and group 3 the Enrofloxacin-treated group (infected and then treated). In addition, each animal was then randomly allocated to one of nine pens. Each pen thus contained four animals from a random assortment of experimental groups.

For the first four days the animals were fed a good quality grass (teff) hay and lucerne hay and provided with water *ad libitum*. Thereafter they were fed a mixed ration containing unmilled lucerne hay, hammer-milled grass hay, yellow maize, hominy chop, cotton seed oil cake meal, begasse, soya beans, salt, sodium bicarbonate and molasses. The ration consisted of approximately 50% roughage and 50% concentrates. Each pen of four animals received 24 kg of the ration per day, i.e. 6 kg per animal per day, divided into two feedings, early morning and late afternoon. Water was provided *ad libitum*.

Challenge infection

For the purposes of this trial, a method of intratracheal challenge infection described by Ames *et al.* (1985) was used. These authors reported that the intratracheal administration of 10^9 colony forming units (CFU) of a 4 h log phase culture of *P. haemolytica* in calves induced clinical signs and macroscopic and microscopic lung lesions indistinguishable from those seen in field outbreaks of pneumonic pasteurellosis.

P. haemolytica A1 (9479) from a field outbreak of pneumonic pasteurellosis in feedlot cattle was selected for use in this trial. This isolate was determined to be sensitive to enrofloxacin using the disc diffusion method (Mastring, Mast Laboratories, Merseyside, United Kingdom).

In a pilot trial, three groups of three animals each were infected intratracheally with 10^6, 10^9 and 10^{12} CFU respectively of a 4 h log phase culture of *P. haemolytica* A1 (9479). In the group which received 10^{12} CFU, lesions typical of pneumonic pasteurellosis were consistently produced, involving $20,5 \pm 1,4\%$ (mean ± SEM) of the total lung volume. It was therefore decided to use a challenge dose of approximately 10^{12} CFU in this trial.

P. haemolytica (9479) was reconstituted in brain/heart infusion (BHI) broth (Oxoid, Unipath Ltd, Basingstoke, United Kingdom) and cultured overnight at 37°C on blood tryptose agar (Biolab Diagnostics, Midrand, South Africa) containing 10% bovine blood to ensure purity. The strain was then cultured overnight at 37°C in BHI broth in four 2 ℓ flasks. The contents of each flask were then inoculated into 20 ℓ of RPMI-1640 medium with L-glutamine (Highveld Biological, Kelvin, South Africa) and cultured for 4 h in a

20 ℓ pilot fermenter (B. Braun and Diessel Biotech GmbH, Melsungen, Germany) to ensure log phase challenge material. This material was then concentrated using a 0,2 µm tangential flow filtration system (Millipore Corp., Bedford, MA) and 2% gelatine was added in order to produce the final challenge material (Ames *et al.* 1985).

Colony counts were performed by taking a 1 mℓ portion of the final material and diluting it ten-fold to 10^{-10}. A 0,1 mℓ volume of each dilution was spread on two blood tryptose agar plates and cultured for 24 h at 37 °C. Viable colonies were then counted and established at approximately 10^{10} CFU per mℓ. The challenge material was thus retrospectively determined to have contained approximately 6×10^{11} CFU per 60 mℓ dose.

After harvesting the challenge material, it was kept at room temperature and administered between 2 and 5 h later. After clipping and aseptic preparation of an area of skin over the cervical trachea, each animal in groups 2 and 3 was inoculated intratracheally with a 60 mℓ dose of the challenge material, using a 60 mℓ plastic syringe and an 18 gauge, 40 mm needle directed down the trachea. During, and for several seconds after injection, each animal's head was held raised to allow the material to flow down the trachea and into the lungs. Each animal in the two infected groups thus received a total dose of 6×10^{11} CFU of *P. haemolytica* A1 intratracheally. The animals in group 1 were kept as non-infected controls. The day on which challenge infection was performed was regarded as day 0.

Treatment

Treatment commenced on the morning of day 2, approximately 40 h post-infection. Each animal in group 3 received 2,5 mg/kg enrofloxacin ("Baytril 5%", Bayer Animal Health, Isando, South Africa) subcutaneously in the neck, once daily for three consecutive days. The animals in group 2 served as untreated controls, each receiving 5 mℓ sterile saline intramuscularly in the neck, once daily for three consecutive days.

Clinical examination

Each of the 36 animals was clinically examined approximately 6 h pre-infection, 18 h post-infection and then every 24 h after that, up to and including day 12. In addition, they were regularly observed in order to detect any animals fulfilling the criteria for euthanasia (see below). In order to ensure consistency in measuring the clinical parameters throughout the trial period, all the examinations were carried out by the same person. The examiner also remained unaware of which treatment each animal had received.

The clinical parameters evaluated at each examination were rectal temperature, habitus (attitude), degree of congestion of ocular mucous membranes and presence of abnormal respiratory sounds on auscultation. The rectal temperature was measured in degrees centigrade using a mercury rectal thermometer. The other three parameters were scored and recorded as shown in Table 1.

TABLE 1 Descriptions of scores assigned to clinical parameters

Parameter	Score	Description
Habitus	0	Normal, alert
	1	Slightly depressed
	2	Moderately depressed
	3	Severely depressed
	4	Moribund (recumbent, unable to rise)
Ocular mucous membrane congestion	0	Normal
	1	Slightly congested
	2	Moderately congested
	3	Very congested
	4	Extremely congested
Lung auscultation	0	Normal
	1	Slight referred bronchial sounds
	2	Moderate referred bronchial sounds
	3	Other abnormal sounds (crackles, wheezes, pleuritic friction rubs)

A decision was made to immediately euthanase any animal which, at any time during the trial period, showed two or more of the following clinical signs: severe congestion of the ocular mucous membranes; severe dyspnoea; protrusion of the tongue and/or extended neck; animal moribund. In such a case, euthanasia was carried out by the intravenous administration of an overdose of pentobarbitone ("Euthanaze", Centaur Laboratories, Silverton, South Africa).

Pathology

On day 14 post infection all the surviving animals were humanely killed and their lungs recovered for further examination. The extent of pneumonic tissue as a percentage of the total lung volume was estimated as described by Jericho & Langford (1982). The extent of consolidated pneumonic tissue was manually plotted onto lung diagrams and then visually estimated as a percentage of each lobe. The person performing this examination was unaware of the treatment received by each animal. According to Jericho & Langford (1982), the percentage of lung mass constituted by each lobe of a normal lung is as follows: left cranial lobe 5%, left posterior cranial lobe 6%, left caudal lobe 32%, accessory lobe 4%, right cranial lobe 6%, right posterior cranial lobe 5%, right middle lobe 7% and right caudal lobe 35%. Using these values and the percentage pneumonic tissue in each lobe, the total percentage of pneumonic

tissue was calculated for each animal and recorded as the lung lesion score.

Samples from representative lesions in two of the cases were collected in 10% buffered formalin and examined histologically.

The lungs of those animals which died or were euthanased before the completion of the trial period were examined immediately after death as described above and the percentage pneumonic tissue was calculated.

Bacteriology

In each case in which macroscopic pneumonic lesions were visible, samples of the affected area were aseptically collected and submitted for aerobic bacterial culture and typing. *P. haemolytica* was identified according to standard methods (Carter 1984) and typed by the method of Biberstein *et al.* (1960).

Statistical methods

Within each of the three groups, the mean, standard deviation and median value of each parameter were calculated for each of the 13 trial days. The rectal temperature of each animal on day 0 was subtracted from each subsequent measurement in order to obtain a "temperature increment" for each day after day 0.

The Wilcoxon signed rank test (Steyn, Smit, Du Toit & Strasheim 1994) was used to determine the day on which the median of each parameter (habitus score, mucous membrane score, lung auscultation score and temperature increment) within each group was no longer significantly different from zero ($P > 0,05$). This indicated the number of days elapsed before each parameter returned to normal.

On each trial day, for each of the parameters, the Kruskal-Wallis test for significant multiple comparisons (Steyn *et al.* 1994) was used in order to detect any significant differences between the group medians ($P \leq 0,05$).

For each parameter and each animal, an area under the curve (AUC) was calculated in order to give a cumulative indication of the severity of disease as reflected by that clinical parameter over the entire trial period. For this purpose the rectal temperature in degrees centigrade was used rather than the temperature increment. The AUC values for each parameter were then compared between groups using the Kruskal-Wallis test.

The lung lesion scores were compared between groups using the Kruskal-Wallis test. Two animals from each group, representing the highest and the lowest lung lesion scores in each group, were excluded from this calculation in order to minimise the effect of these outliers.

The animals which died or were euthanased before the completion of the trial period were included in all calculations up until the time of death. Their lung lesion scores were included with the others in the calculations.

RESULTS

Clinical examination

A graphical representation of the mean of each parameter within each group is shown in Fig. 1–4. By 18 h post-challenge, marked elevations were recorded in the four clinical parameters amongst the animals in the two infected groups. They became listless and anorexic, with many of them showing a serous nasal discharge and occasionally a cough. Thereafter, the severity of the disease, as reflected by each clinical parameter, declined gradually over the next several days. Two animals, both in group 2, died, one on day 2 and the other on day 3. A third animal, in group 3, was euthanased on day 4 because it was suffering from severe respiratory distress, with extended neck and open-mouth breathing. By day 12, most of the surviving animals in the two infected groups were again bright and alert and had regained their normal appetite. The animals in group 1 showed very little change in the four clinical parameters measured, and throughout the trial period remained bright and alert with a normal appetite.

With the exception of rectal temperature (Fig. 1), the parameters of the animals in group 2 were significantly higher than those of group 1 virtually throughout the trial period ($P \leq 0,05$). The same parameters of the animals in group 3 were significantly higher than those of group 1 for the first four to six days post-infection ($P \leq 0,05$), but thereafter were generally no longer significantly different from those of group 1 ($P > 0,05$).

Significant differences ($P \leq 0,05$) between the parameters of the animals in the enrofloxacin-treated group and those of the untreated (Positive Control) group were only very occasionally detected. The small increases in temperature recorded in all three groups on day 6, 9 and, to a lesser extent, 12 (Fig. 1), were probably due to the clinical examinations having taken place 2–3 h later in the morning than usual. Corresponding increases in the other parameters on those dates were not seen.

The temperature increment of group 3 subsided to a value not significantly different from zero on day 4, whereas for group 2 this happened on day 5. The habitus scores reached zero on day 9 for group 3 and on day 10 for group 2. The lung auscultation score of group 3 reached zero on day 8 whereas that of group 2 remained significantly higher than zero for the duration of the trial period. The mucous membrane

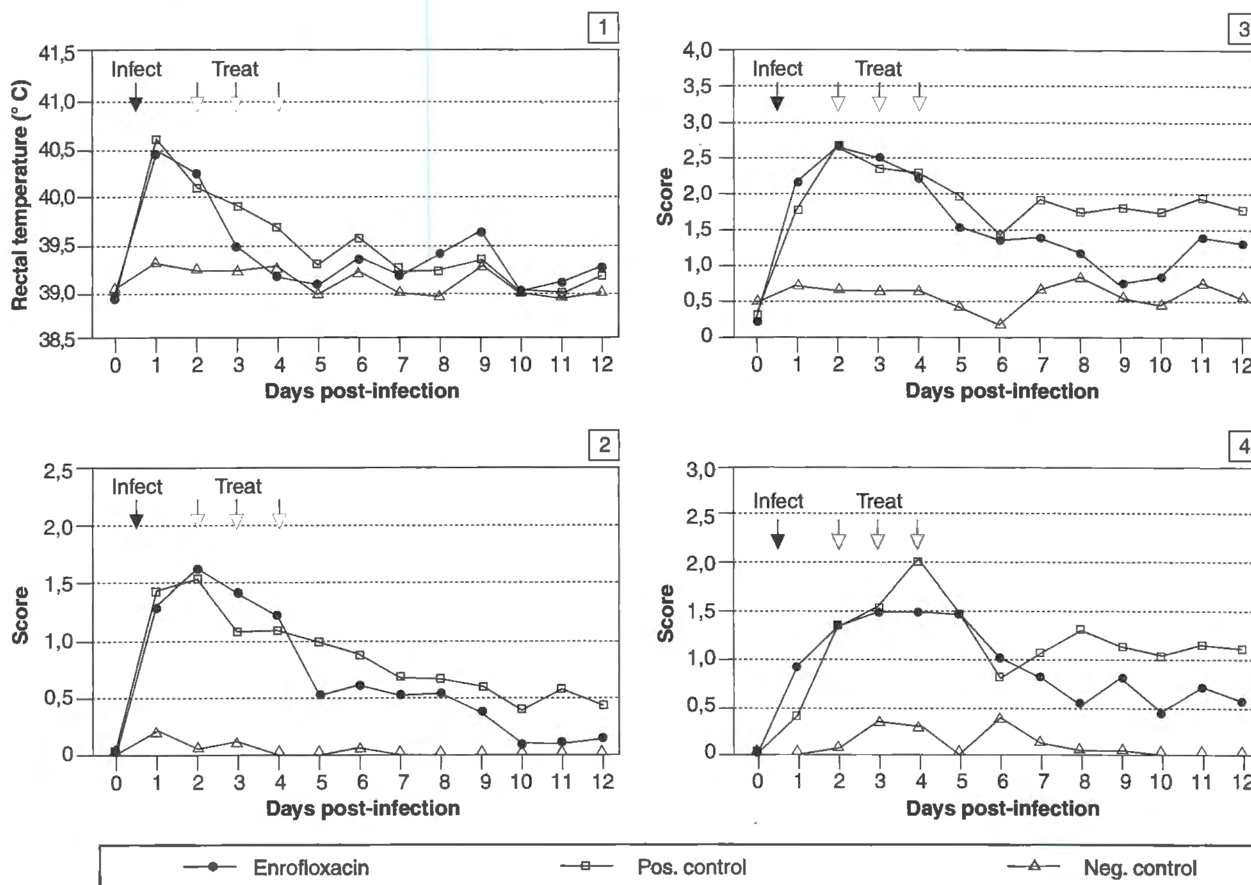

FIG. 1 Mean daily rectal temperatures of calves infected intratracheally with *Pasteurella haemolytica* A1 and treated for three days with 2,5 mg/kg enrofloxacin; calves infected and not treated (Positive Control); and non-infected calves (Negative Control)

FIG. 2 Mean daily habitus scores of calves infected intratracheally with *Pasteurella haemolytica* A1 and treated for three days with 2,5 mg/kg enrofloxacin; calves infected and not treated (Positive Control); and non-infected calves (Negative Control)

FIG. 3 Mean daily ocular mucous membrane congestion scores of calves infected intratracheally with *Pasteurella haemolytica* A1 and treated for three days with 2,5 mg/kg enrofloxacin; calves infected and not treated (Positive Control); and non-infected calves (Negative Control)

FIG. 4 Mean daily lung auscultation scores of calves infected intratracheally with *Pasteurella haemolytica* A1 and treated for three days with 2,5 mg/kg enrofloxacin; calves infected and not treated (Positive Control); and non-infected calves (Negative Control)

congestion score of neither group returned to zero during the trial period. All the parameters of the animals in group 1 remained not significantly different from zero for most of the trial period.

The mean values for the areas under the curve (AUC) are shown in Table 2. Although the AUC values of group 3 were noticeably lower than those of group 2, no significant differences were found ($P > 0,05$).

Pathology

The lungs of the three animals which died or were euthanased before completion of the trial showed severe acute bronchopneumonia with severe fibrinous pleuritis, typical of field cases of acute pneumonic pasteurellosis. The lesions involved respectively 41,6%, 49,7% and 64,5% of the total lung volume. The lungs of the surviving 33 animals, examined after slaughter on day 14, were either macroscopically normal or showed a wide variation in lesion size, the largest involving 36,3% of the total lung volume. The lesions were characterized as subacute fibrinopurulent bronchopneumonia with a variable degree of fibrinous pleuritis present in many cases. Lung lesions from the two animals that were examined histologically were consistent with subacute pneumonic pasteurellosis and included necrotic alveolitis, streaming neutrophils, oedema and fibrin exudation.

No macroscopically visible lung lesions were found in any of the animals in group 1. The mean lung lesion scores for groups 2 and 3 were 12,1% and 8,4% respectively, but this difference was not statistically significant ($P > 0,05$) (Table 2).

TABLE 2 Mean cumulative (AUC) values for rectal temperature (Temp), habitus score (Hab), mucous membrane score (MM) and lung auscultation score (Lung), and mean lung lesion scores (Lesion) following intratracheal challenge with *Pasteurella haemolytica* A1

Group	Temp	Hab	MM	Lung	Lesion
Negative Control	469 (0,5)^{a}	0,6 (0,2)^{a}	7,0 (1,4)^{a}	1,5 (0,5)^{a}	0 (0)^{a}
Positive Control	474 (1,0)^{b}	9,6 (1,3)^{b}	22,6 (1,5)^{b}	13,9 (1,6)^{b}	12,1 (4,7)^{b}
Enrofloxacin	473 (1,0)^{b}	8,2 (1,2)^{b}	18,2 (1,9)^{b}	11,1 (1,1)^{b}	8,4 (3,1)^{b}

Values in parentheses are standard errors of the mean (SEM)
Values in the same column with differing superscripts differ significantly ($P \leq 0,05$)

Bacteriology

Aerobic bacterial culture of all the cases in which pneumonic lesions were found ($n = 21$) yielded pure cultures of *P. haemolytica* A1 in 16 cases (76%); *P. haemolytica* and *Actinomyces pyogenes* in three cases (14%); *P. haemolytica* and *Proteus mirabilis* in one case (5%); and *P. mirabilis* and *Morganella morganii* in one case (5%). Of these isolates, *P. mirabilis* and *M. morganii* were considered to be sample contaminants.

DISCUSSION

In this trial, the successful induction of clinical pneumonic pasteurellosis was confirmed by the demonstration at the end of the trial period of both macroscopic and microscopic pulmonary lesions typical of pneumonic pasteurellosis and by the isolation of *P. haemolytica* A1 from these lesions. The disease was not transmitted to the group 1 (Negative Control) animals despite their close contact with the infected animals. Members of each experimental group were randomly distributed between pens rather than kept together in their groups in order to reduce the effect of any environmental variables related to any particular pens.

The large variation in the severity of disease and the extent of lesions produced in the experimentally infected animals limited our ability to detect differences between the two infected groups. One of the reasons for this may have been a variation between animals in the amount of challenge material actually reaching the alveoli after intratracheal injection using a 40 mm needle. In the pilot trial referred to under "Materials and Methods", the challenge material was administered through a 6 gauge dog urinary catheter inserted into the trachea via the lumen of a 12 gauge canula, thus ensuring that the bacterial broth was deposited close to the bronchial openings. This could perhaps be expected to produce more consistent pulmonary lesions. Another possible reason for the variation in severity of disease may have been a variation in the specific immune status of the calves prior to experimental infection. The calves had originated from a farm with no history of respiratory disease, had been reared as a single group on pasture, had not been exposed to animals from other sources and had received no vaccinations against respiratory disease. However, it was not known whether any of them possessed significant specific immunity to *P. haemolytica* A1, as their antibody titres were not determined in this trial. Concurrent viral respiratory infections at the time of challenge may possibly have resulted in the development of more severe pneumonia in certain animals, but no obvious signs of respiratory infection were seen in any animals prior to challenge. Although randomisation should on average have equalised the groups, the above factors may have affected our ability to detect significant differences between groups.

Of the bacterial virulence factors which are implicated in the pathogenesis of pneumonic pasteurellosis, the ones that have received the most attention are leukotoxin and lipopolysaccharide (endotoxin) (Whiteley *et al.* 1992). Leukotoxin is a protein exotoxin produced by *P. haemolytica* only during the logarithmic growth phase, which induces lysis of bovine alveolar macrophages and neutrophils within the alveoli. Thus it initiates the cycle of neutrophil chemotaxis and degranulation which is responsible for the severe lung pathology. Lipopolysaccharide readily crosses the alveolar wall and, via several mechanisms including activation of the complement pathways, has a systemic inflammatory effect. The changes in habitus, rectal temperature, mucous membrane congestion score and lung auscultation score which were seen in this trial may be ascribed to a large extent to the release of endotoxin as the bacteria are killed (Cullor & Smith 1996). This would have occurred in both the Positive Control and the enrofloxacin-treated groups as bacteria were killed either by natural body defence systems or by the exogenous antibiotic, and this may partly explain the lack of clear, clinically observable differences between the two groups. The commencement of treatment, 40 h after infection, may also have been too late to prevent the development of severe pulmonary lesions in some animals, resulting in extensive lesions being observed in some of the enrofloxacin-treated animals at the end of the trial period. Inflammatory lesions with neutrophil accumulation have been described 4 h after intratracheal inoculation of *P. haemolytica* in calves (Whiteley, Maheswaran, Weiss & Ames 1991). The early development of

lesions in the course of the disease thus probably necessitates an earlier onset of treatment than was used in this trial.

Although significant differences were seldom detected between the enrofloxacin-treated animals and the positive control animals, very commonly the situation occurred in which the value of a particular parameter in the Positive Control group was significantly higher than that of the Negative Control group whereas the value in the enrofloxacin-treated group was not. Examples of this were on days 5–9 for habitus score and on days 10–12 for both mucous membrane and lung auscultation scores. Thus it appears that treatment with enrofloxacin had a beneficial effect on the clinical course of the disease in this trial. In the field, an additional benefit which may be expected with the use of an effective antibiotic would be a decrease in the number of viable bacteria excreted during the treatment period, thus lowering the bacterial challenge to susceptible animals.

The cumulative value for each parameter, expressed as the area under the curve (AUC), may be expected to give the best overall indication of the efficacy of the drug in reducing the effects of the disease as reflected by that parameter. The value of each individual parameter for assessment of the severity of disease is, however, not known.

The extent of lung involvement in pneumonic pasteurellosis should have an influence on the animal's ability to perform under feedlot conditions after apparent clinical recovery from the disease. It is likely that the extent of the lung lesions recorded after slaughter on day 14 was a good indication of their extent immediately following the course of treatment, as it has been reported that the extent of the pulmonary lesions on days 2–3, day 6 and days 9–10 post-infection in experimental pneumonic pasteurellosis is very similar (Allan, Gibbs, Wiseman & Selman 1985). Treatment with enrofloxacin resulted in a lower mean lung lesion score than did no treatment, although this difference was not statistically significant ($P > 0{,}05$).

ACKNOWLEDGEMENTS

The authors wish to thank Mr Hennie Prinsloo for assistance in preparing the challenge material, Mrs Ariane Neethling for advice regarding statistical methods and for processing the data and Dr John Jardine and Prof. N.P.J. Kriek for doing the histopathological examinations.

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