Efficacy of Enrofloxacin against Severe Experimental Anaplasma marginale Infections in Splenectomized Calves

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INTRODUCTION

Anaplasmosis, caused by the rickettsial hemoparasite Anaplasma marginale, is one of the most prevalent tick-transmitted diseases of cattle worldwide.1–3 Persistent infections of A. marginale restrict the export of animals to areas where the disease is not regarded as endemic. In a review article, Kocan and associates3 estimated the cost of anaplasmosis in the United States to be more than $300 million per year.

Before the development of imidocarb dipropionate and the tetracycline antimicrobials, a variety of chemotherapeutic agents, including arsenicals, antimalarials, antimony derivatives, and dyes, were used to treat acute anaplasmosis. These compounds had little if any chemotherapeutic effect.4 Chlortetracycline and oxytetracycline are the only compounds approved for use against acute anaplasmosis in the United States. Anaplasma infections are not sterilized at the usual recommended therapeutic doses of the tetracycline antimicrobials.5,6 There are currently no antimicrobials labeled for the elimination of persistent anaplasmosis infections in carrier animals.

Splenectomized calves have been used as a model for evaluating the efficacy of antimicrobials against A. marginale.6–9 Successful clear-
ance of anaplasmosis in splenectomized calves has not been achieved with either imidocarb or oxytetracycline,6,9 perhaps because serum drug concentrations achieved with these products are inadequate or because of the absence of an adequate immune response to work in combination with the antimicrobial to eliminate the parasite.10

Enrofloxacin (Baytril, Bayer Animal Health) is a fluoroquinolone antimicrobial that inhibits bacterial DNA gyrase (topoisomerase II) and topoisomerase IV.11 This activity prevents DNA supercoiling and decatenation of original chromosomes and replicates. Two published reports indicate that enrofloxacin is effective against acute A. marginale infections at dose rates of 5 to 10 mg/kg.12,13 However, the dynamics of the infection following treatment and the potential for elimination of the carrier state have not been assessed. The need for additional trials to study whether enrofloxacin is useful to control severe anaplasmosis infections has been recognized.13

The purpose of this study was to test the efficacy of enrofloxacin against experimental A. marginale infection in splenectomized calves. The specific aims were to evaluate the efficacy of enrofloxacin administered subcutaneously to severely infected animals (>25 percent parasitized erythrocytes [PPE]) twice, 48 hours apart, at a rate of 12.5 mg/kg. In addition, it was our intent to study the dynamics of A. marginale infection following treatment to ascertain whether this antimicrobial could be efficacious against persistent A. marginale infections caused by a variety of isolates as might be encountered in field outbreaks of the disease.

**MATERIALS AND METHODS**

This protocol was approved by the Iowa State University (ISU) Committee on Animal Care (COAC).

**Experimental Cattle**

Five Holstein calves were obtained from the ISU Dairy Breeding Research facility (Ankeny, IA), and one animal was purchased from northwest Iowa (Alton, IA). Both herds had no recorded cases of anaplasmosis. Individual animal details are summarized in Table 1. Calves were approximately 150 to 240 days old and weighed between 68 and 169 kg at the time of treatment. Animals were confirmed to be free of A. marginale antibodies by competitive ELISA (cELISA; Anaplasma Antibody Test Kit, VMRD [Veterinary Medical Research & Development], Pullman, WA).14,15 Splenectomies were performed when calves were ap-

<table>
<thead>
<tr>
<th>Calf ID</th>
<th>Isolate</th>
<th>Infection to Peak PPE (days)</th>
<th>Peak PPE</th>
<th>Dose of Enrofloxacin (mg)</th>
<th>Amount Administered (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Oklahoma</td>
<td>39</td>
<td>55.50</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>63</td>
<td>St. Maries</td>
<td>19</td>
<td>43.30</td>
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</tr>
<tr>
<td>71</td>
<td>St. Maries</td>
<td>25</td>
<td>53.20</td>
<td>850</td>
<td>9</td>
</tr>
</tbody>
</table>

PPE = percent parasitized erythrocytes.
approximately 3 months old using the technique described by Thompson and associates.\textsuperscript{16}

**Housing and Husbandry**

Calves were housed in a biolevel 2 livestock infectious disease isolation facility at ISU. They were housed in pairs and fed approximately 1.5 kg of a maize-based ration twice daily. The ration was manufactured without the addition of tetracycline antibiotics. Animals also received a small amount of grass hay twice daily and water ad libitum for the duration of the study.

**Experimental Infection with Anaplasmosis**

Approximately 6 to 8 weeks after splenectomy was performed, two calves were subinoculated with blood from a carrier animal experimentally infected with a West Coast (St. Maries) isolate of \textit{A. marginale}. Three calves were similarly infected with blood and a blood stabilate infected with an Oklahoma isolate of \textit{A. marginale}. The sixth calf received blood stabilate containing a Virginia isolate of \textit{A. marginale}.

**Postinfection Monitoring**

After being infected, the calves were monitored daily for the clinical signs of anaplasmosis, including anorexia, depression, and listlessness. Blood samples were collected at least once weekly after infection for determination of PPE, packed cell volume (PCV), and cELISA serology. Blood samples were collected by jugular venipuncture using 18-gauge, 1-inch needles (Air-Tite Products, Virginia Beach, VA). For serum collection, 10-ml Monoject No Additive Sterile glass tubes (Sherwood Medical, St. Louis) were used. For whole blood (PCV and PPE), 7-ml K$_2$EDTA glass tubes (BD Vacutainer, BD Diagnostics, Franklin Lakes, NJ) were used. Blood in EDTA was refrigerated before PCV testing or packaged in insulated material for overnight delivery by courier to Oklahoma State University for determination of the PPE.

Blood smears for PPE determination were stained using a 30-second, three-step technique (Hema 3 Manual Staining System, Fisher Scientific, Pittsburgh, PA) comparable to the Wright–Giemsa method. Two slides were prepared for each blood sample and examined for the presence of \textit{A. marginale} under oil immersion at 1,000× magnification using a grid. A total of 500 cells was counted within the four squares of the grid, and the number of infected cells was recorded. The PPE was determined according to the equation above.

Serologic testing by cELISA was conducted by the ISU Veterinary Diagnostic Laboratory. The test was conducted in accordance with the method described in the Office International des Epizooties \textit{Manual of Diagnostic Tests and Vaccines for Terrestrial Animals}\textsuperscript{17} and the manufacturer’s recommendations.\textsuperscript{14} Results are given as percent inhibition. PCVs were determined by partially filling heparinized capillary tubes (Chase Scientific Glass, Rockwood, TN) with blood; the tubes were then centrifuged for 3 minutes using an Adams Micro-Hematocrit centrifuge (Model CT 2900, Clay Adams, New York).

**Treatment**

Treatment with enrofloxacin was initiated 19 to 25 days after experimental infection, when the PPE ranged from 28.6 to 53.2. Each treated animal received two subcutaneous injections of enrofloxacin administered 48 hours apart at a dose rate of 12.5 mg/kg. Control animals did not receive any treatment. US federal law prohibits the extralabel use of enrofloxacin in food-producing animals. Accordingly, experimental

\[
PPE = \frac{\text{No. of Infected Cells}}{\text{Total No. of Cells Counted}} \times 100
\]
animals were kept in an isolation facility after treatment and were euthanized and incinerated at the end of the study.

Posttreatment Monitoring

Blood samples were collected at least once weekly for approximately 42 days after treatment for PPE, PCV, and cELISA serology.

Statistics

Data were entered into a spreadsheet program (Excel 2003, Microsoft, Redmond, WA) for subsequent calculation and manipulation. The mean ± SEM (standard error of the mean) was calculated for all parameters. Hypothesis tests were conducted using JMP 5.1.2 analytical software (SAS Institute, Cary, NC). Group differences between treated and control animals were analyzed using a Student’s t-test. Statistical significance was designated a priori as a P value ≤ .05.

PPE results after treatment were analyzed using a simple linear regression model. The null hypothesis was that treatment with enrofloxacin had no effect on PPE over time after treatment. This hypothesis was tested using a Student’s t-test of the slope of the linear regression line to determine if the gradient was 0. An analysis of variance (ANOVA) F-test for comparing the simple linear regression model to a separate means (one-way ANOVA) model was used to assess the goodness of fit of simple linear regression. A large P value (> .05) indicated that there was no evidence of lack-of-fit to the simple linear regression model.

In addition to the linear regression modeling, one-way ANOVA tests were conducted to determine whether there were statistical differences between days after treatment. This approach was preferred when there was evidence of lack-of-fit to the linear regression model. The Tukey–Kramer honest significant difference test was used to compare the mean PPE, PCV, and cELISA each day to identify statistical differences between days after enrofloxacin therapy. This test is an exact α-level test if the sample

<table>
<thead>
<tr>
<th>TABLE 2. Summary of the Mean Percent Parasitized Erythrocytes (PPE) and Packed Cell Volume (PCV) after Administration of Enrofloxacin (12.5 mg/kg SC twice, 48 hours apart)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Days after Treatment</strong></td>
</tr>
<tr>
<td><strong>Value</strong></td>
</tr>
<tr>
<td><strong>Mean PPE</strong></td>
</tr>
<tr>
<td><strong>SEM</strong></td>
</tr>
<tr>
<td><strong>Lower 95% CI</strong></td>
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<tr>
<td><strong>Upper 95% CI</strong></td>
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<td><strong>Mean PCV</strong></td>
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<tr>
<td><strong>SEM</strong></td>
</tr>
<tr>
<td><strong>Lower 95% CI</strong></td>
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<tr>
<td><strong>Upper 95% CI</strong></td>
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<tr>
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<tr>
<td><strong>6</strong></td>
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<tr>
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<td><strong>18</strong></td>
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<td><strong>30</strong></td>
</tr>
<tr>
<td><strong>36</strong></td>
</tr>
<tr>
<td><strong>42</strong></td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Entries with different superscript letters are significantly different (P < .01).

SEM = standard error of the mean.
sizes are the same and conservative if the sample sizes are different.\textsuperscript{21}

\section*{RESULTS}

Following infection, animals in the treated and control groups demonstrated a similar increase in PPE and decrease in PCV ($P = .39$ and .72, respectively). Both animals in the control group became moribund when the PCV dropped below 10\% (normal PCV, 24\% to 46\%); this occurred approximately 3 to 6 days after peak parasitemia, and the calves were euthanized. At this time, the difference in PPE between the treatment and control groups was approaching statistical significance ($P = .061$).

At the time of treatment (21 ± 1.35 days after infection), the splenectomized calves had a mean (±SEM) rickettsemia of 39.13 (±5.67) PPE (range, 28.6 to 53.2 PPE). The mean PCV was 26.45\% (±3.02\%), and the cELISA was 82.66 (±3.879) percent inhibition. By 6 days after treatment, the mean PPE had decreased by 62 to 14.88 (±4.64). The mean PCV had also decreased by 48\% to 13.80\% (±2.17\%). The mean PPE remained between 3.60 (±2.34) and 4.98 (±4.78) for the next 18 days, with levels fluctuating for individual animals. Recrudescence of rickettsemia, however, was self-limiting, and further treatment was not required. A progressive increase in mean PCV was generally observed from 6 days after treatment to the end of the study. The mean (±SEM) cELISA ranged from 80.06 (±3.3) percent inhibition on day 7 to 89.44 (±0.93) percent inhibition on day 42. These differences were not statistically significant. The PPE and PCV results are summarized in Table 2 and represented graphically in Figure 1.

Linear regression of PPE against days post-treatment provided substantial evidence that the slope of the regression line was not zero ($P < .0002$). The lack-of-fit $F$-test ($P = .0028$), however, indicated that variability between the group means for all the data could not be explained by a simple linear regression model. A one-way ANOVA was conducted and provided substantial evidence ($P < .0001$) of posttreatment PPE differences. The Tukey–Kramer honest significant difference test indicated that only the PPE at treatment was significantly different ($P < .001$).

Based on these findings, the PPE measurements taken over the first 12 days were subjected to simple linear regression. This is similar to the approach used by Kuttler and Simpson.\textsuperscript{5} The equation of the regression line was determined to be:

\[
PPE = 29.875 - 3.196 \times \text{days after first treatment}
\]

A lack-of-fit $F$-test indicated that a regression line was appropriate to describe these data.
Linear regression provided substantial evidence to reject the null hypothesis that treatment with enrofloxacin had no effect on PPE over time based on the slope of the regression line ($P < .0002$). Instead, these data supported the alternative hypothesis that enrofloxacin substantially reduced the PPE over time after treatment.

Similar simple linear regression analyses were also conducted on the PCV and cELISA results. There was evidence of lack-of-fit, indicating that a one-way ANOVA test provided a more appropriate analysis. Despite the large fluctuation in PCV observed during the first 2 weeks of the study, there was no evidence of a statistically significant difference in PCV and cELISA in treated animals over the course of the study ($P > .05$).

**DISCUSSION AND CONCLUSION**

The purpose of this study was to evaluate the efficacy of enrofloxacin against severe (>25 PPE) experimental *A. marginale* infections in splenectomized calves. In addition, our goal was to assess whether enrofloxacin could clear persistent *A. marginale* infections. This study found that treated animals survived severe infections, whereas untreated calves became moribund and had to be euthanized. In surviving animals, there was substantial evidence that two doses of enrofloxacin (12.5 mg/kg) administered 48 hours apart suppressed but did not eliminate severe *A. marginale* infections in splenectomized calves. A recrudescence of *A. marginale* parasites was observed in all animals within 30 days after treatment. Subsequent rickettsemia was less severe and self-limiting.

The PCV returned to pretreatment levels within 6 weeks after treatment.

Because of the lack of qualified subjects and the cost of the animals, only six calves were enrolled in this study, thereby limiting the ability to extrapolate the results. The control calves became moribund and had to be euthanized when the PCV dropped below 10%; at this point, the PPE exceeded 40, and failure to intervene on welfare grounds could not be justified. Previous studies have indicated that severe anaplasmosis infection in splenectomized calves is fatal if left untreated. In the present study, the control animals had to be euthanized because of clinical anaplasmosis, necessitating the use of a simple linear regression model to analyze data from the remainder of the study.

The efficacy of enrofloxacin against acute *A. marginale* infections has been studied in two previous reports. Schröder et al. examined the effect of enrofloxacin on *A. marginale* in three trials. Splenectomized and intact calves were treated with two injections at a lower dose (either 5 or 10 mg/kg) of enrofloxacin when the PCV decreased to 25%. Either dose rate was deemed effective based on the disappearance of parasites from blood smears and the return of PCVs to pretreatment levels. However, these researchers did not determine whether enrofloxacin eliminated persistent infections, which are characterized by parasitemia that is not microscopically detectable in intact animals.

Guglielmone and associates treated intact Holstein steers with enrofloxacin at a dosage of 10 mg/kg for 2 consecutive days when the PPE ranged from 3 to 10 (a significantly lower level of parasitemia than was noted in our study).

Anaplasmosis in the United States is estimated to cost more than $300 million per year.
Following treatment, a significantly lower rickettsemia \( (P < 0.01) \) was detected at 72 hours; however, animals were not evaluated for recrudescence of rickettsemia or infectivity following treatment. Furthermore, the disease described in both these studies was not as severe as the infection model we used.

The value of splenectomized calves in the study of chemotherapeutic agents against \textit{A. marginale} has been described in the literature. Splenectomized calves have been used to emphasize the drug effect and minimize possible immune responses that might camouflage specific drug actions. Furthermore, splenectomized calves present an obvious advantage in early screening trials because of the consistent relapse pattern occurring in instances in which treatment is ineffective.\(^{22}\) Roby\(^{8}\) demonstrated increased survival of splenectomized calves treated parenterally with imidocarb at 2.5, 5.0, and 10.0 mg/kg. Kuttler\(^{9}\) observed a pattern of parasite suppression and recrudescence after administration of imidocarb dipropionate (5 mg/kg) and subsequently controlled by variant-specific primary immune responses.\(^{24,25}\) These cycles are microscopically undetectable in intact animals.\(^{26}\) In contrast, microscopically detectable cycles occur in splenectomized animals that survive primary infection, presumably due to the absence of phagocytosis of parasitized erythrocytes in the spleen, which would ordinarily remove parasitized erythrocytes in intact animals.\(^{27}\) In previously published reports, it is noteworthy that subsequent cycles appear more frequently but are self-limiting in splenectomized calves after therapy. This observation could not be confirmed in our study because the animals were monitored for only one cycle after treatment. Recrudescences with higher levels of rickettsemia might be observed over a longer interval if extensive antigenic variation occurred in major surface protein (MSP)-2/MSP-3.

In the United States, federal law restricts the use of enrofloxacin in cattle to treatment of bovine respiratory disease infections associated with \textit{Mannheimia haemolytica}, \textit{Pasteurella multocida}, and \textit{Histophilus somni} (\textit{Haemophilus somnus}) only. Flexible dosing allows single-dose therapy at 7.5 to 12.5 mg/kg SC or multiday therapy at 2.5 to 5 mg/kg SC q24h for 3 days. The bactericidal activity of fluoroquinolone antimicrobials is dependant on the ratios of the area under the plasma drug concentration curve (AUC) to the minimum inhibitory concentration (MIC) for the organism.\(^{28}\) Enrofloxacin is metabolized to ciprofloxacin in calves, which contributes between 10% and 27% of the total concentration present in the serum.\(^{29}\) The MICs of enro-

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\textbf{There was substantial evidence that two doses of enrofloxacin suppressed but did not eliminate severe \textit{A. marginale} infections in splenectomized calves.}
Floxacin and ciprofloxacin against *A. marginale* have not been determined. Antimicrobial susceptibility studies involving ciprofloxacin have been conducted on *Anaplasma phagocytophilum*, a closely related agent responsible for human granulocytic anaplasmosis. These studies have determined the MIC of ciprofloxacin to be between 1 and 2 µg/ml. We therefore administered enrofloxacin twice, 48 hours apart, at a dose rate of 12.5 mg/kg to maximize the AUC above the hypothesized MIC.

Our study examined three geographically and phylogenetically distinct isolates of *A. marginale*. These isolates were selected based on work conducted by de la Fuente and associates, who demonstrated that the Virginia isolate belonged to a distinct southeastern clade of *A. marginale* and the St. Maries isolate to a distinct western clade. This also resembles the heterologous situation likely to be encountered in field application of antimicrobial therapy to a diverse population of animals. It is noteworthy that Maurin and colleagues demonstrated differences in the MIC of the fluoroquinolone levofloxacin against different geographic isolates of *A. phagocytophilum*. In vitro MICs varied from 0.06 to 0.5 µg/ml. The latter concentration is reported to be close to the maximum levels achievable in human serum. Our very limited study did not show any evidence of a difference in susceptibility of these *A. marginale* isolates to enrofloxacin in vivo. However, this finding requires further evaluation before a definitive conclusion can be made.

In an earlier study, Maurin and associates demonstrated a DNA gyrase–mediated natural resistance to fluoroquinolones in *Ehrlichia chaffeensis* and *Ehrlichia canis*. This corresponds to a single amino acid difference in *gyrA*, which encodes the A subunit of DNA gyrase in *A. phagocytophilum*. Concern has been expressed that acquired fluoroquinolone resistance in *A. phagocytophilum* could be expected with widespread use. The recent publication of the complete genome sequencing of *A. marginale* may allow DNA sequence alignment of the quinolone resistance–determining region (QRDR) of *gyrA*. This would be helpful assessing potential differences in quinolone susceptibility between *A. marginale* and *A. phagocytophilum* as well as the potential for quinolone resistance should these compounds become widely used.

The results of the present study indicate that administration of two doses of enrofloxacin (12.5 mg/kg SC) given 48 hours apart ameliorates severe *A. marginale* infections in splenectomized calves. Untreated control animals became moribund and were euthanized. Recovering animals remained persistently infected after treatment with this regimen. Further studies are warranted to investigate whether a dose regimen of enrofloxacin can be identified to eliminate *A. marginale* infection from carrier cattle.

**REFERENCES**


