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Flow cytometric evaluation of selected antimicrobial efficacy for clearance of *Anaplasma marginale* in short-term erythrocyte cultures

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The tick-borne rickettsia, *Anaplasma marginale*, causes the economically important cattle disease anaplasmosis. Once infected, cattle remain lifelong carriers. Herein, we used flow cytometry to test the efficacy of three antimicrobials; oxytetracycline, imidocarb and enrofloxacin against Virginia (VGN) or Oklahoma (OK) *A. marginale* isolates in short-term erythrocyte cultures. Parasite viability was assessed using the vital dye hydroethidine (HE), which is detectable when living organisms convert HE to ethidium bromide. Viability of *A. marginale* in selected cultures was determined by subinoculation into susceptible calves. Data were analyzed by MANOVA, Tukey-Kramer honest significant difference and Wilcoxon rank sum tests. Receiver operating characteristic (ROC) analysis was used to correlate results with culture infectivity. Enrofloxacin inhibited *A. marginale* in a dose dependent manner. Surprisingly, higher concentrations of imidocarb were less effective than lower concentrations against *A. marginale* with significant differences ($P < 0.05$) observed between the two isolates. Oxytetracycline was the least active drug tested. Cultures infected with the OK isolate exposed to 4.0 μg/mL enrofloxacin and those of the VGN and OK isolates exposed to 1.0 μg/mL imidocarb were sterilized. This is the first *in vitro* study demonstrating the efficacy of enrofloxacin against *A. marginale*. Furthermore, these data indicate that flow cytometry is a useful assay for screening antimicrobials against *A. marginale*.

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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 (Uilenberg, 1995; Dumler *et al.*, 2001; Kocan *et al.*, 2003). Chlortetracycline and oxytetracycline are, thus far, the only compounds approved for use against acute anaplasmosis in the United States. However, *A. marginale* infections are not sterilized at the recommended therapeutic doses of tetracycline (Kuttler & Simpson, 1978; Stewart *et al.*, 1979). Furthermore, tetracycline formulations currently marketed for control of anaplasmosis in cattle are not approved for the elimination of persistent infections.

Successful antimicrobial therapy against *A. marginale* depends on (i) achieving adequate drug concentrations at the site of infection, (ii) ensuring that drug concentration is maintained for a sufficient duration to be effective, (iii) the susceptibility of the organism to the antimicrobial, (iv) pharmacokinetic parameters of the drug and (v) the local environment as reviewed by Bidgood and Papich (2003). Susceptibility tests facilitate the determination of a minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial agent which prevents visible growth of a microorganism in an agar or broth dilution susceptibility test [National Committee for Clinical Laboratory Standards (NCCLS) 2002]. The application of susceptibility testing to include the intra-cellular parasites such as rickettsial pathogens has been complicated by the requirement of a cell culture system for propagation of these organisms.

Kessler *et al.* (1979) developed a whole blood culture of *A. marginale* based on a similar method for cultivation of *Plasmodium*. Viability of cultures from days 13 and 33 was demonstrated by inoculation and infection of susceptible calves. Subsequently, Wyatt *et al.* (1991) described a flow cytometry method for evaluating the growth and viability of cultured intraerythrocytic protozoan hemoparasites. This assay utilized the

selective uptake and metabolic conversion of hydroethidine (HE) to ethidium bromide (EB) by live parasites in intact erythrocytes. The intercalation of EB, a DNA binding fluorochrome, into viable parasites allowed the use of fluorescence activated cell sorting (FACS) to distinguish between erythrocytes containing viable organisms and those containing dead or no parasites. Studies with the hemoparasite *Babesia bovis* utilized this fluorochrome technique to monitor the effect of parasiticidal drugs on parasites in whole erythrocyte cultures.

The purpose of this study was to utilize whole infected erythrocyte culture and FACS to evaluate the effect of oxytetracycline, imidocarb and enrofloxacin against *A. marginale* isolates collected from two different geographic locations, Oklahoma (OK) and Virginia (VGN). The percent reduction in infected erythrocytes as detected by HE staining was used to select cultures for inoculation into calves. This facilitated the determination of minimum rickettsiacidal concentrations (MRCs) defined as the lowest concentration of an antimicrobial agent which rendered *A. marginale* infection in erythrocyte cultures noninfectious thereby indicating successful antimicrobial sterilization.

MATERIALS AND METHODS

Propagation of A. marginale in splenectomized calves

Two Holstein calves were purchased from the Iowa State University Dairy Breeding Research facility at Ankeny, IA. Animals were confirmed free of *A. marginale* antibodies by use of a competitive enzyme linked immunoabsorbent assay (<30% inhibition, cELISA *Anaplasma* Antibody Test Kit, VMRD Inc. Pullman, WA, USA); (Torioni De Echaide *et al.*, 1998). Splenectomies were performed when calves were approximately 3 months old using the technique described by Thompson *et al.* (1992).

Approximately 6–8 weeks after splenectomy, one calf was infected with 10 mL of blood stabilate containing an OK isolate with 31.6 percent parasitized erythrocytes (PPE). The second calf received 10 mL of blood stabilate containing a VGN isolate with 35.5% parasitized erythrocytes. Blood stabilates were prepared from packed erythrocytes washed in phosphate-buffered saline (PBS) and frozen in liquid nitrogen at a 1:1 ratio with PBS containing 10% dimethylsulphoxide (DMSO). Stabilates were maintained in liquid nitrogen and dry ice prior to inoculation. This protocol was approved by the Committee on Animal Care (COAC) at Iowa State University. The design of this study is outlined graphically in Fig. 1.

Postinfection monitoring

Blood samples were collected once weekly postinfection to determine PPE and packed cell volume (PCV). Blood in EDTA was refrigerated prior to 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-sec, three-step staining technique (Hema 3® Staining System, Fisher Scientific, Pittsburgh, USA) comparable with the Wright-Giemsa method. Two slides were prepared for each blood sample and examined for the presence of *A. marginale* under oil immersion at 1000× magnification using a grid. A total of 500 cells were counted within the four squares of the grid and the number of infected cells was recorded. PPE = (number of infected cells/total number of cells counted) × 100.

PCVs were determined by partially filling heparinized capillary tubes (Chase Scientific Glass INC, Rockwood, TN, USA) with blood and spun for 3 min using an Adams Micro-Hematocrit centrifuge (Model CT 2900; Clay Adams INC, New York, NY, USA).

Preparation of erythrocyte cultures

Blood was collected aseptically in heparin from splenectomized calves when the PPE was 28.6% and 31.4% for animals inoculated with the VGN and OK isolate, respectively. Whole blood was centrifuged at 600 ***g*** at 4 °C for 15 min and the plasma and buffy coats were removed. Cells were resuspended and washed twice in RPMI 1640 (Cellgro, Mediatech, Inc. Herndon, VA, USA) culture medium at 4 °C (Kessler *et al.*, 1979).

Drug susceptibility assays were performed in 96-well culture microplates in the presence of a final erythrocyte concentration of 10% (vol/vol) and serial dilutions of drugs. Forty-eight wells on each plate were inoculated with blood infected with the VGN isolate with the remainder inoculated with the OK isolate. Cell culture medium comprised RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 25 mM HEPES buffer, 200 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Controls consisted of plates containing erythrocytes infected with each isolate without the addition of the antimicrobials to be tested and noninfected bovine erythrocytes.

Antimicrobial agents

Stock solutions of antimicrobials at a concentration of 1 mg/mL were prepared in cell culture medium. These stock solutions were used to prepare the following antimicrobial dilutions: Oxytetracycline hydrochloride (OTC; Sigma-Aldrich, St Louis, MO, USA) at 1; 2; 4; 8 and 16 μg/mL; imidocarb (IMD; N,N′-Bis[3-(4,5-dihydro-1H-imidazol-2-yl)phenyl]urea dipropanoate, 99%; Sigma-Aldrich) at 0.25; 0.5; 1; 2 and 4 μg/mL and enrofloxacin (ENRO; Fluka, Buchs, Switzerland) at 0.25; 0.5; 1; 2 and 4 μg/mL. A volume of 180 μL of each dilution was added to each well. Following addition of 20 μL of washed packed erythrocytes, cultures were incubated at 37 °C and 5% CO_2 for 7 days.

Cell preparation and hydroethidine staining

On each study day, three wells from each isolate and drug concentration, including the control cultures, were washed in separate centrifugation tubes containing 1 mL 1X PBS. Samples were pelleted by centrifugation for 5 min at 450 ***g*** at 4 °C.

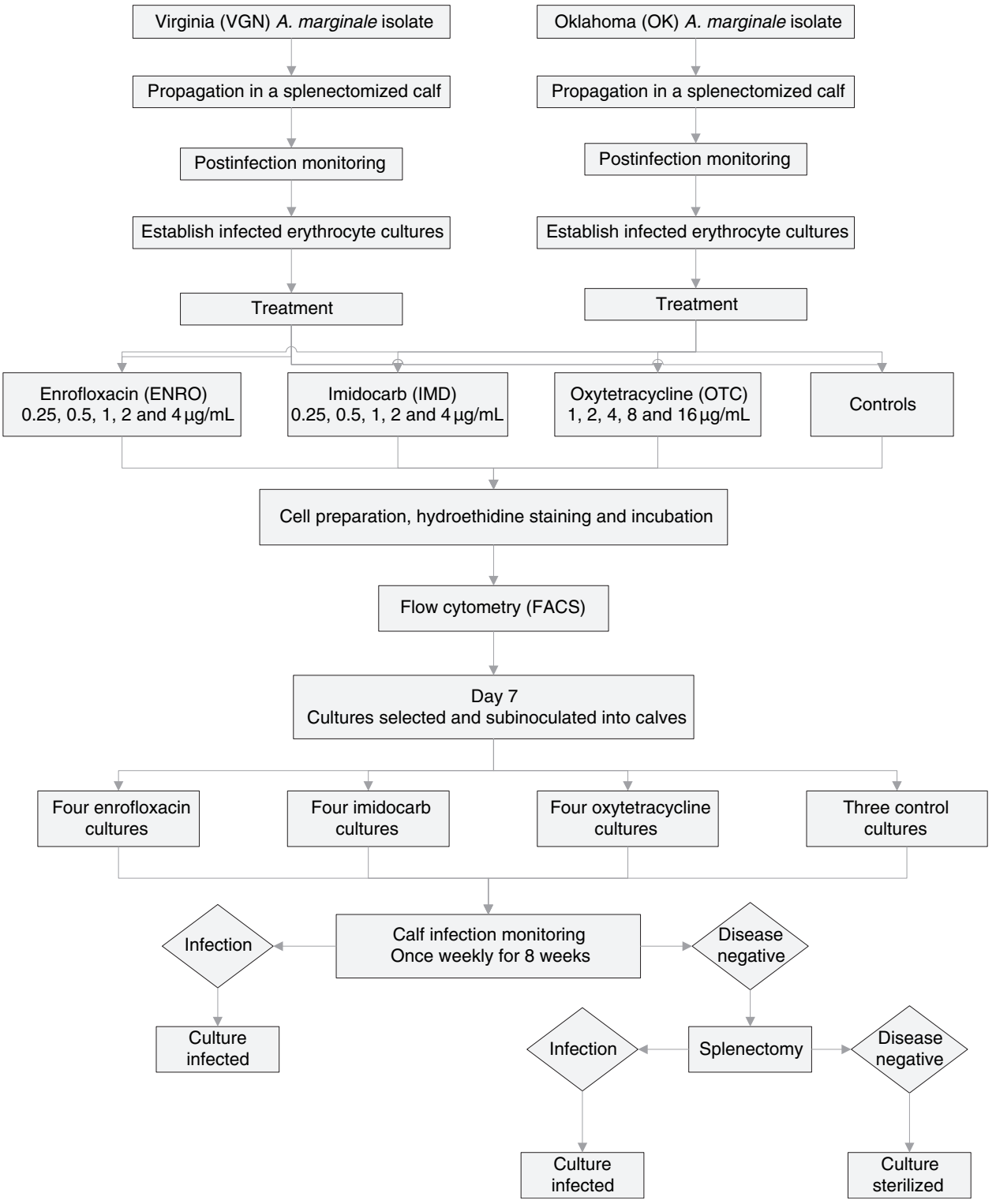

Fig. 1. Flow chart depiction of the study design. Parasitized erythrocytes were harvested from splenectomized calves infected with either a Virginia or Oklahoma *A. marginale* isolate. Cultures were established in 96 well plates and treated with doubling dilutions of enrofloxacin, imidocarb or oxytetracycline or remained as untreated controls. Following 7 days of culture and flow cytometric analysis (FACS) to detect conversion of hydroethidine to ethidium bromide by viable parasites, two cultures from each isolate/antimicrobial combination were selected for inoculation into calves. Sterilization of *A. marginale* infections in culture was confirmed by establishing that calves remained free of anaplasmosis for 8 weeks after intra-venous inoculation of cultured erythrocytes and for an additional 4 weeks after splenectomy.

Cell staining for FACS was performed using a method similar to that described by Wyatt and others (1991). Briefly, dihydroethidium (Hydroethidine; Molecular Probes, Eugene, OR, USA) was solubilized in anhydrous DMSO at 1 mg/mL as a stock solution. Fifteen microgram solubilized HE was diluted in 1 mL PBS and added to the pelleted erythrocytes. Suspensions were incubated in the absence of light for 30 min at 37 °C, 5% CO_2. Following incubation, 980 μL of suspension was removed and replaced with 1 mL PBS. Diluted samples were centrifuged for 5 min at 450 ***g*** at 4 °C. Pelleted erythrocytes were resuspended in 1 mL FACS buffer containing 0.1% bovine serum albumin and 1 X PBS. Unused HE in DMSO was frozen at −20 °C in 1 mL aliquots and thawed at 37 °C prior to use.

Flow cytometric analysis

Cell suspensions to be analyzed by FACS were diluted with PBS to obtain a flow rate of 2000–5000 fluorescent events/second. A total of 150 000 events were counted daily for each of the three wells harvested from culture. An inclusion gate was set based on the forward scatter and side scatter characteristics of the HE-treated uninfected erythrocyte control. The suspensions were analyzed by argon-ion laser fluorescence excitation at 488 nm and emission at 585 nm (range 563–607 nm; FL-2) in log Fl 2 data mode using a Becton-Dickinson FACScan and CellQuest computer software (BD Biosciences, San Jose, CA, USA). Fluorescent profiles were recorded for later analysis using FlowJo computer software (Tree Star, Inc. Ashland, OR, USA).

Testing infectivity of selected cultures

The infectivity of parasitized erythrocytes following 7 days of culture was determined from samples selected on the basis of the FACS results. The contents of 20 wells (4000 μL) from each selected sample were pelleted by centrifugation at 450 ***g*** for 5 min. Approximately 400 μL packed cells were resuspended in 1.5 mL RPMI 1640. Samples were stored on ice prior to IV inoculation into intact Holstein calves.

Fifteen Holstein calves were confirmed free of *A. marginale* antibodies by two consecutive competitive ELISA (cELISA) tests (Anaplasma Antibody Test Kit; VMRD Inc. Pullman, WA, USA). Resuspended erythrocytes were inoculated intravenously by jugular injection using a 16G, 1 inch needle. Two calves received untreated infected control culture suspensions while one calf received the uninfected control suspension. The remaining 12 calves received selected treated samples. Four calves were randomly assigned to each antimicrobial treatment with two pairs receiving the OK and VGN isolate, respectively.

Following inoculation, calves were monitored daily for clinical signs of anaplasmosis, including anorexia, depression and listlessness. Blood samples were collected at least once weekly for approximately 8 weeks postinfection for PPE, PCV and cELISA serology as described previously. After 8 weeks, calves failing to demonstrate signs of anaplasmosis were splenectomized and monitored for an additional 4 weeks. Cultures exposed to antimicrobial concentrations that failed to cause disease following subinoculation into calves that were splenectomized were deemed sterilized. These drug concentrations were considered rickettsiacidal after 7 days. Those cultures in which infectivity was retained were deemed treatment failures.

Statistical analyses

Data were entered into a spreadsheet program (Excel 2003; Microsoft Corporation, Redmond, WA, USA) for subsequent calculation and manipulation. The mean ± SEM (standard error of the mean) EB positive cell count was calculated from the three wells from each culture analyzed each day. The percent reduction in EB positive cells was determined as the mean number of EB positive (infected) cells in the test culture subtracted from the mean number of EB positive (infected) cells in the control culture divided by the total number of EB positive control cells, multiplied by 100.

Hypothesis tests were conducted using JMP 5.1.2 analytical software (SAS Institute, INC, Cary, NC, USA). FACS data were analyzed using repeated measures analysis. The null hypothesis was that the percent reduction in EB positive cells was zero. The analysis of variance approach to repeated measures data was used based on a review by Everitt (1995). The Wilk's Lambda test was selected to evaluate within group interactions and evidence of time × group interactions which uses a likelihood ratio statistic for testing that a multivariate contrast is zero, assuming multivariate normality and further assuming equality of covariance matrices across groups (Everitt & Dunn, 2001). In all analyses the Wilk's Lambda test indicated a statistically significant interaction ($P < 0.001$). This significant interaction was expected as it indicates that percent reduction in EB positive cells following exposure to each antimicrobial treatment and dilution was not parallel over time. Consequently, it was concluded that exposure to each antimicrobial and antimicrobial dilution resulted in different patterns of *A. marginale* suppression over the course of 7 days.

Accordingly, differences between antimicrobials and antimicrobial dilutions were analyzed using ANOVA and the Tukey-Kramer HSD (honest significant difference) method for multiple comparisons. This test is an exact alpha concentration test if the sample sizes are the same and conservative if the sample sizes are different (Hayter, 1984). Statistical significance was designated *a priori* as a P-value ≤ 0.05. Differences between isolates exposed to a particular drug concentration were analyzed using Wilcoxon rank sum tests (chi-square approximation) as these data were not normally distributed.

In order to determine the percent EB positive cells which provided the optimum sensitivity and specificity in predicting sterilization of anaplasmosis infections in culture, we conducted receiver-operating characteristic (ROC) analysis on each day of the study (Greiner *et al.*, 2000). An ROC curve is a plot used to assess the quality of the discriminatory power of a test using sensitivity and specificity data. Constructing an ROC curve allows the assessment of how well a test discriminates between positive and negative results and allows the identification of the best trade-off between sensitivity and specificity when evaluating

a test. The underlying assumption of ROC analysis in the present study were that the diagnostic variable (EB positive cells detected by FACS) could be used to discriminate between two mutually exclusive states; in this case infectivity (as determined by PPE and cELISA >30%) or noninfectivity of test cultures following inoculation into calves. A ROC curve is a plot of sensitivity by (1 – specificity) for each percent reduction value. The area under the ROC curve (AUC) is a common index used to summarize the information contained in the curve. In the case of a perfect test where sensitivity and specificity are both 100%, the AUC would be 1.

RESULTS

Detection of parasitized erythrocytes

Differences in HE conversion were detected between uninfected and infected erythrocytes incubated with HE and analyzed by flow cytometry. Representative FACS histograms of HE treated samples are shown in Fig. 2. Cells in uninfected cultures did not convert HE to EB as demonstrated by a single population of cells or a single histogram peak. Parasitized cells in infected cultures converted HE to EB and appeared as a

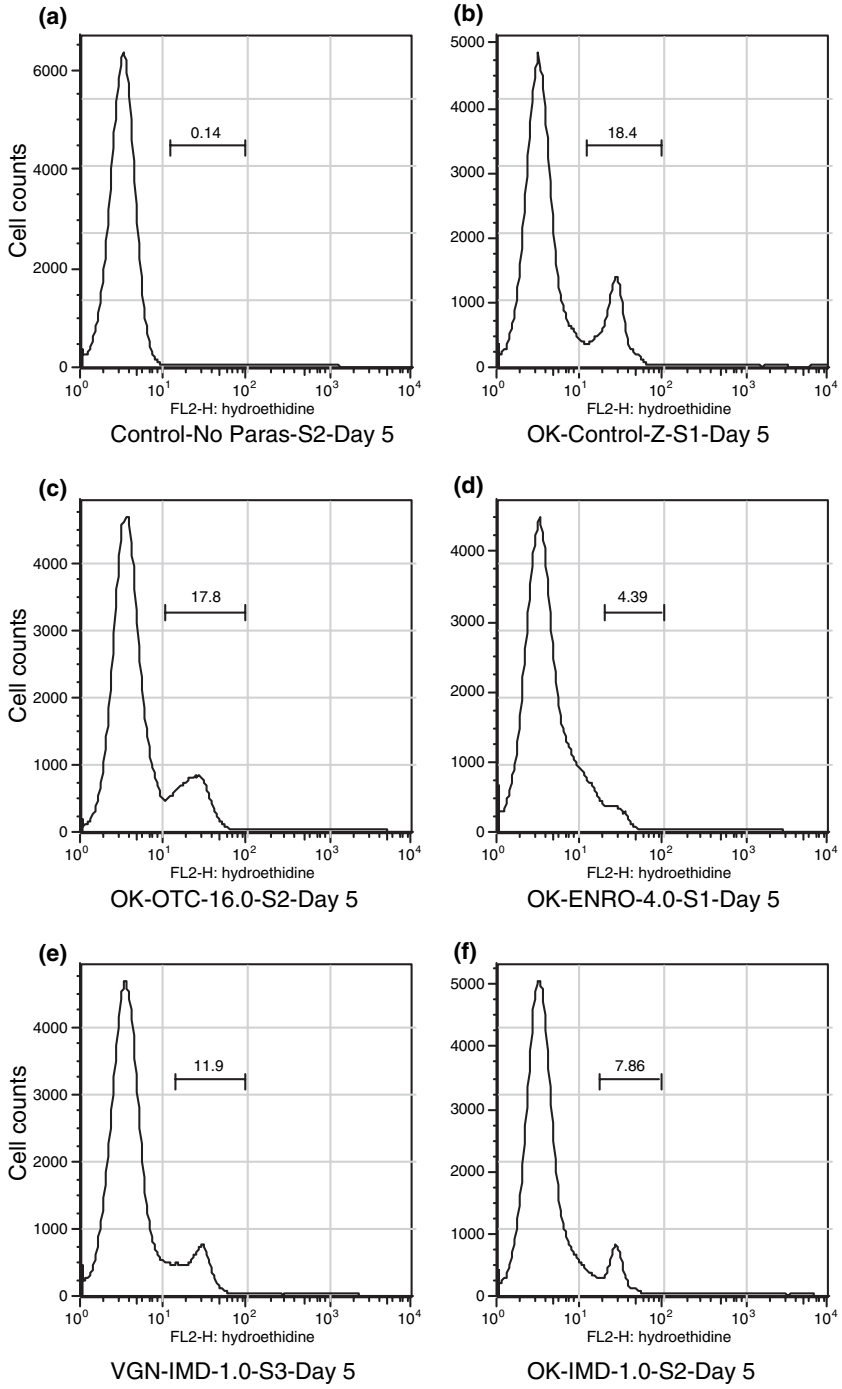

Fig. 2. Flow cytometry histograms demonstrating viable *A. marginale* as labeled by ethidium bromide (EB). Cells in uninfected cultures did not convert hydroethidine (HE) as demonstrated by a single histogram peak/population (a). Parasitized cells in infected cultures converted HE to EB and appeared as a second population (18.4%) with a log shift in mean fluorescent intensity as compared with uninfected cells in untreated cultures (b). Selected Oklahoma (OK) and Virginia (VGN) isolate cultures exposed to 16 μg/mL oxytetracycline (OTC) (c); 4 μg/mL enrofloxacin (ENRO) (d) and 1 μg/mL imidocarb (IMD) (e) and (f) for 5 days are presented. Samples (d)–(f) failed to infect calves after 7 days of culture.

second population of cells or a second peak on the histogram with a log-increase in mean fluorescent intensity from uninfected cells.

The number of EB positive cells in parasitized untreated control cultures increased two- to threefold over the first 3 days of culture for both isolates (results not shown). Thereafter the number of fluorescent cells progressively decreased suggesting a reduction in HE conversion to EB. This is consistent with the findings of Kessler *et al.* (1979) who reported that PPE detected microscopically using Giemsa-stained blood smears doubled during the first 3 days of culture and then gradually decreased before peaking again after 8 days. Infectivity of cultures was confirmed in both the present and previously published report by subinoculation into calves.

Differences in mean percent reduction in EB positive cells between antimicrobials

Significant differences were observed in the mean percent reduction in EB positive, parasitized erythrocytes between the enrofloxacin, imidocarb and oxytetracycline treated cultures (ANOVA $P < 0.05$; Table 1). Comparisons for all pairs of treatments using Tukey-Kramer HSD indicated that the mean (±SEM) percent reduction after 24 h was greater in the oxytetracycline (30.02% ± 2.71% reduction) and enrofloxacin (23.54% ± 2.00% reduction) treated cultures compared with the imidocarb (11.24% ± 1.23% reduction) culture ($P < 0.01$). On day 2 and day 6 there were fewer parasitized erythrocytes in the enrofloxacin treated cultures when compared with the imidocarb treated cultures and significantly more parasites in the oxytetracycline treated cultures. On the remaining days of the study there was no difference between enrofloxacin and imidocarb treated cultures but more parasitized cells in the oxytetracycline treated cultures.

Differences in mean percent reductions between antimicrobial dilutions

The mean percent reduction in EB positive cells for each antimicrobial dilution by isolate are presented in Table 1. The results for each antimicrobial test are summarized as follows:

Enrofloxacin

The mean percent reduction in EB positive cells increased with increasing antimicrobial concentrations in cultures exposed to enrofloxacin for 7 days when data from both isolates was pooled (Fig. 3). Over the course of the study, cultures exposed to 0.5–4 μg/mL enrofloxacin had a greater mean percent reduction in parasitized cells when compared with cultures exposed to 0.25 μg/mL enrofloxacin ($P < 0.05$). In comparison with the other antimicrobials, cultures exposed to 0.5–4 μg/mL enrofloxacin had significantly fewer parasitized erythrocytes than cultures exposed to 0.25 μg/mL imidocarb and 1–16 μg/mL oxytetracycline ($P < 0.05$).

Imidocarb

A paradoxical increase in EB positive cells was observed in cultures exposed to 4 μg/mL imidocarb for 7 days when data from both isolates was pooled (Fig. 4). Cultures exposed to 1 and 2 μg/mL imidocarb had significantly greater percent reduction in parasitized cells when compared with cultures exposed to 0.5 and 4 μg/mL imidocarb ($P < 0.05$). These cultures in turn had fewer EB positive cells than cultures exposed to 0.25 μg/mL imidocarb. In comparison with the

Table 1. Comparison between the mean reduction in ethidium bromide (EB) positive erythrocytes following exposure to enrofloxacin (ENRO), imidocarb (IMD) and oxytetracycline (OTC) in whole blood cultures for 7 days

Drug/concentration	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Mean	SEM
ENRO 0.25 μg/mL	18.97	21.53^{a}	14.05^{b}	7.21^{b}	17.26^{b}	34.30^{c}	11.17^{b}	17.78^{b}	3.76
ENRO 0.5 μg/mL	24.75	24.86^{a}	24.61	26.90	37.79	44.45^{a}	43.98^{a}	32.48^{a}	3.62
ENRO 1 μg/mL	21.48	30.47^{a}	29.11^{a}	36.70^{a}	47.68^{a}	50.57^{a}	29.25	35.03^{a}	5.39
ENRO 2 μg/mL	24.88	31.12^{a}	27.56	32.82^{a}	44.16^{a}	45.49^{a}	35.56^{a}	34.51^{a}	3.69
ENRO 4 μg/mL	27.61	32.80^{a}	34.29^{a}	42.69^{a}	61.68^{a}	58.98^{a}	55.53^{a}	44.80^{a}	5.97
ENRO mean	23.54^{a}	28.15^{a}	25.92^{a}	29.26^{a}	41.71^{a}	46.76^{a}	35.10^{a}	32.92^{a}	4.48
IMD 0.25 μg/mL	16.99	9.53^{b}	12.82^{b}	16.23	21.41^{b}	22.65	4.62^{b}	14.89^{b}	3.11
IMD 0.5 μg/mL	10.14^{b}	15.96	31.96^{a}	23.44	30.02	39.73^{c}	24.38	25.09^{c}	4.61
IMD 1 μg/mL	13.05^{b}	23.00^{a}	45.13^{a}	44.38^{a}	44.86^{a}	42.26^{a}	43.78^{a}	36.64^{a}	5.14
IMD 2 μg/mL	5.53^{b}	28.06^{a}	40.33^{a}	43.37^{a}	49.18^{a}	44.58^{a}	39.35^{a}	35.77^{a}	5.70
IMD 4 μg/mL	10.51^{b}	21.04^{a}	28.79^{a}	31.71^{a}	35.37	29.31	21.38^{b}	25.44^{c}	3.52
IMD mean	11.24^{b}	19.52^{b}	31.81^{a}	31.82^{a}	36.17^{a}	35.71^{b}	26.70^{a}	27.57^{a}	4.42
OTC 1 μg/mL	35.27^{a}	2.40^{b}	13.10^{b}	0.65^{b}	4.78^{b}	3.06^{b}	0.00^{b}	8.46^{b}	5.19
OTC 2 μg/mL	29.08^{a}	0.98^{b}	8.74^{b}	5.63^{b}	5.95^{b}	0.64^{b}	0.00^{b}	7.29^{b}	3.43
OTC 4 μg/mL	38.59^{a}	1.74^{b}	8.82^{b}	0.00^{b}	7.67^{b}	0.00^{b}	0.00^{b}	8.12^{b}	5.54
OTC 8 μg/mL	23.64	2.37^{b}	5.08^{b}	0.00^{b}	4.07^{b}	8.37^{b}	1.25^{b}	6.40^{b}	3.10
OTC 16 μg/mL	23.52	5.66^{b}	9.41^{b}	0.00^{b}	17.97^{b}	6.86^{b}	2.14^{b}	9.37^{b}	3.43
OTC mean	30.02^{a}	2.63^{c}	9.03^{b}	1.26^{b}	8.09^{b}	3.79^{c}	0.68^{b}	7.92^{b}	4.14

For each day of the study, the mean percent reduction in EB positive erythrocyte values designated with different superscript letters are significantly different (Tukey-Kramer HSD $P < 0.05$).

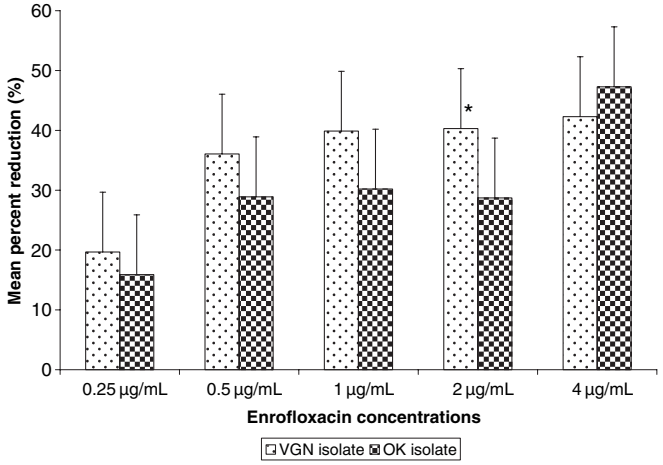

Fig. 3. Comparison between the mean percent reduction in hydroethidine positive erythrocytes determined by FACS in cultures containing the Virginia (VGN) or Oklahoma (OK) isolate of *Anaplasma marginale* following exposure to enrofloxacin (ENRO) for 7 days. Asterisks indicates a significant difference in mean percent reduction between isolates at each antimicrobial dilution (Wilcoxon test $P < 0.05$).

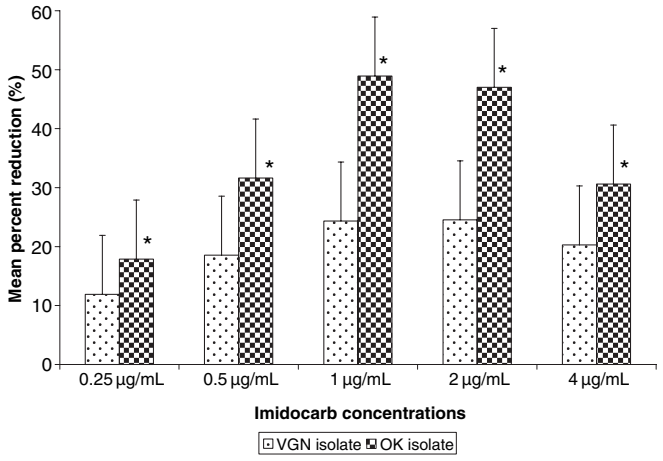

Fig. 4. Comparison between the mean percent reduction in hydroethidine positive erythrocytes determined by FACS in cultures containing the Virginia (VGN) or Oklahoma (OK) isolate of *Anaplasma marginale* following exposure to imidocarb (IMD) for 7 days. Asterisks indicates a significant difference in mean percent reduction between isolates at each antimicrobial dilution (Wilcoxon test $P < 0.05$).

other antimicrobials, cultures exposed to 1 and 2 µg/mL imidocarb had significantly fewer parasitized erythrocytes than cultures exposed to 0.25 µg/mL enrofloxacin and 1–16 µg/mL oxytetracycline ($P < 0.05$).

Oxytetracycline

Significant differences were not observed between the mean percent reductions in parasitized erythrocytes and increasing antimicrobial dilutions in cultures exposed to oxytetracycline for 7 days when data from both isolates was pooled (Fig. 5). The greatest reduction in EB positive cells occurred within the first 24 h of exposure to 1–4 µg/mL oxytetracycline. In comparison

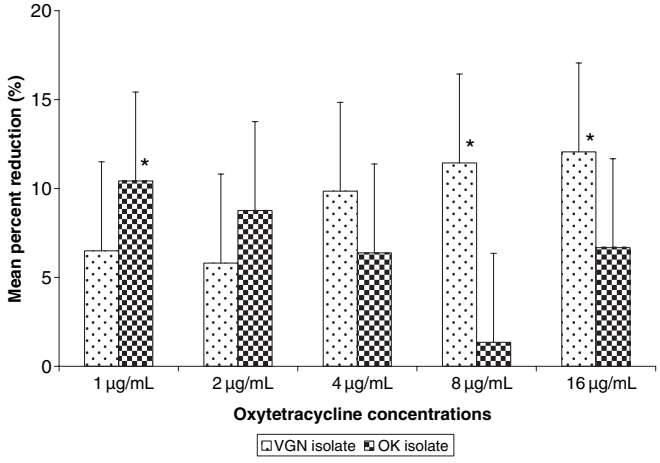

Fig. 5. Comparison between the mean percent reduction in hydroethidine positive erythrocytes determined by FACS in cultures containing the Virginia (VGN) or Oklahoma (OK) isolate of *Anaplasma marginale* following exposure to oxytetracycline (OTC) for 7 days. Asterisks indicates a significant difference in mean percent reduction between isolates (Wilcoxon test $P < 0.05$).

with the other antimicrobials, cultures exposed to oxytetracycline had a significantly lower percent reduction in EB positive cells.

Differences between isolates

Considerable variation in the percent reduction in EB positive cells occurred over the 7 day period of the study and statistical differences were noted at various drug dilutions on several days. The mean percent reduction in EB positive cells for each isolate is presented in Figs 3–5.

Enrofloxacin

A significantly greater mean percent reduction in EB positive cells was observed in the VGN isolate cultures as compared with the OK cultures exposed to 2 µg/mL enrofloxacin (40.31% ± 2.36% compared with 28.72% ± 1.96% ($P = 0.0007$) for 7 days.

Imidocarb

Significant differences between isolates were observed in cultures exposed to 0.25–4 µg/mL imidocarb. In all cases, a greater reduction in parasitized cells occurred in cultures infected with the OK isolate. This ranged from 30.61% ± 2.66% in the OK isolate culture compared with 20.28% ± 1.44% reduction in the VGN isolate culture exposed to 4.0 µg/mL imidocarb ($P = 0.0006$) to 48.93% ± 3.97% in the OK culture compared with 24.34% ± 1.95% reduction in the VGN culture exposed to 1.0 µg/mL imidocarb ($P = 0.0001$).

Oxytetracycline

There was significantly greater percent reduction in EB positive cells in the VGN isolate cultures when compared with the OK cultures exposed to 8 and 16 µg/mL oxytetracycline ($P < 0.05$). The opposite was found in cultures exposed to 1 µg/mL

oxytetracycline where there were fewer parasites in the OK treated cultures ($P = 0.04$).

Testing infectivity of selected cultures

Based on the percent reduction in EB positive cells collected by FACS, we selected pairs of cultures infected with the VGN and OK isolates treated with 0.25 and 4.0 μg/mL enrofloxacin, 0.25 and 1.0 μg/mL imidocarb and 1.0 and 16.0 μg/mL oxytetracycline for inoculation into calves. Cultures containing uninfected blood and those infected with the OK isolate exposed to 4.0 μg/mL enrofloxacin and the VGN and OK isolate exposed to 1.0 μg/mL imidocarb failed to elicit a serological response or parasitemia in calves (cELISA <30% inhibition). Following splenectomy, infection was not detected in these calves. The other sub-inoculated calves developed a parasitemia and seroconverted within 10–31 days postinoculation.

Comparison between FACS and in vivo infectivity

The results of the ROC analysis are summarized in Table 2 and Fig. 6. The area under the ROC curve ranged from 0.67 on day 1 to 0.95 on day 3. On day 1, the ability of FACS to detect infection in culture (sensitivity) was 47% and ability of FACS to detect sterilization of culture (specificity) was 91% at a cut-off value of 7.89% EB positive cells. This increased to a sensitivity of 88% and a specificity of 86% at a cut-off of 14% EB positive cells on day 3.

DISCUSSION AND CONCLUSIONS

The purpose of this study was to use whole blood culture and FACS to evaluate the effect of enrofloxacin, imidocarb and oxytetracycline against an OK and VGN isolate of *A. marginale*. The percent reduction in EB positive cells was used to select cultures for inoculation of calves in order to determine the infectivity of the culture. Sterilization of *A. marginale* infections in culture was confirmed by determining that calves remained free of anaplasmosis for 8 weeks after intravenous inoculation of cultured erythrocytes and for an additional 4 weeks after splenectomy since removal of the spleen would elicit latent infections within 14 days (Foote *et al.*, 1951). We propose the term MRC to describe the lowest concentration of antimicrobial which sterilizes *A. marginale* infections in cell culture. This is more accurate and descriptive than the term minimum inhibitory concentration (MIC) which is commonly used to describe the lowest antimicrobial concentration which prevents visible growth of a bacteria in agar or broth dilution susceptibility tests (NCCLS, 2002). Based on these data we concluded that the MRC of enrofloxacin against the OK isolate of *A. marginale* was >0.25 μg/mL but ≤ 4.0 μg/mL. However the MRC of enrofloxacin against the VGN isolate was >4.0 μg/mL. In contrast the MRC of imidocarb against both isolates was between 0.25 μg/mL and 1.0 μg/mL. The MRC of oxytetracycline required to be effective against anaplasmosis is >16.0 μg/mL.

Table 2. Summary of the receiver operating characteristic (ROC) analysis used to determine the percent EB positive cells which most closely correlated with sterilization of anaplasmosis infections in culture on each day of the study

Study day	Area under ROC curve (AUC)	Cut-off value (EB positive cells) (%)	Sensitivity (%)	Specificity (%)
1	0.67	7.89	47	91
2	0.78	16.20	60	95
3	0.95	14	88	86
4	0.89	11.70	80	100
5	0.86	12.10	73	100
6	0.79	7.69	68	100
7	0.85	6.15	77	100

A receiver operating characteristic curve (ROC) is a plot of sensitivity by (1 – specificity) for each percent reduction in EB positive cells. The area under the ROC curve (AUC) is a common index used to summarize the information contained in the curve. In the case of a perfect test the AUC would be 1. At the cut-off value presented, the curve of the ROC plot can be used to determine the ability of FACS to detect infection in culture (sensitivity) and sterilization of culture (specificity).
AUC-noninformative (AUC = 0.5), less accurate (0.5 < AUC ≤ 0.7), moderately accurate (0.7 < AUC ≤ 0.9), highly accurate (0.9 < AUC < 1.0) and perfect tests (AUC = 1) (Swets, 1988).

The data presented suggest that flow cytometry is a reliable predictor of infectivity, although infectivity varied between days of the study. The area under the ROC curve ranged from 0.67 on day 1 to 0.95 on day 3. ROC analysis has been increasingly used for the evaluation of clinical laboratory tests although its use in medical and veterinary literature is limited (Greiner *et al.*, 2000). The area under the ROC curve is a global summary statistic of diagnostic accuracy. According to an arbitrary guideline suggested by Swets (1988), these data can distinguish between noninformative (AUC = 0.5), less accurate (0.5 < AUC ≤ 0.7), moderately accurate (0.7 < AUC ≤ 0.9), highly accurate (0.9 < AUC < 1.0) and perfect tests (AUC = 1). Based on these guidelines the optimum correlation between FACS data and infectivity occurred on day 3 when the AUC was 0.95. These data suggest that future susceptibility studies using this method may be conducted over 3 as opposed to 7 days as in the present study.

Our results indicated that enrofloxacin inhibits *A. marginale* in a dose dependent manner. Two published reports indicated that enrofloxacin (Baytril®, Bayer Animal Health) was effective against acute *A. marginale* infections *in vivo* at dosages of 5–10 mg/kg (Schröder *et al.*, 1991; Guglielmone *et al.*, 1996). To our knowledge the present study is the first report to evaluate a minimum effective concentration of enrofloxacin against *A. marginale*. These data, in conjunction with published pharmacokinetic and pharmacodynamic information, may facilitate an assessment of the potential use of enrofloxacin against persistent *A. marginale* infections.

Antimicrobial susceptibility studies involving ciprofloxacin, an active metabolite of enrofloxacin, have been conducted against *Anaplasma phagocytophilum* which causes human granulocytic anaplasmosis (HGE). When this organism is grown in the human promyelocytic cell line, the MIC of ciprofloxacin is between 1 and 2 μg/mL (Klein *et al.*, 1997; Branger *et al.*, 2004). The present study examined two geographically and

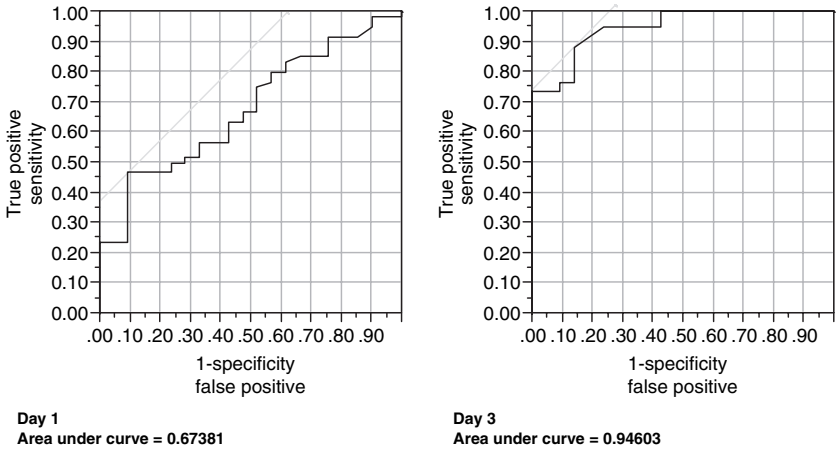

Fig. 6. Receiver operating characteristic (ROC) curves from day 1 and 3 used to assess the ability of flow cytometric analysis of parasitized, ethidium bromide positive erythrocytes to discriminate between two mutually exclusive states; namely infectivity or noninfectivity of treated cell cultures following inoculation into calves, using sensitivity and specificity data.

phylogenetically distinct isolates of *A. marginale* as previously reported by de la Fuente *et al.* (2001). These data revealed statistical difference between the two isolates exposed to 2 μg/mL enrofloxacin for 7 days. However, only the OK isolate exposed to 4.0 μg/mL was not infective when injected into an intact calf. Maurin *et al.* (2003) 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 which is reported to be close to the maximum concentrations achievable in human serum. Our study suggested that similar susceptibility differences may exist between the OK and VGN isolates of *A. marginale* but the exact mechanism still remains to be determined. This finding may limit the widespread use of enrofloxacin in field cases of *A. marginale*. Furthermore, Federal (USA) law also prohibits the extra-label use of enrofloxacin in food producing animals.

Our study revealed a statistical difference in the percent reduction in EB positive cells between the OK and VGN *A. marginale* isolates at all the imidocarb dilutions tested. Roby and Mazzola (1972) found that two injections of imidocarb dipropionate, administered at 5 mg/kg 14 days apart, eliminated *A. marginale* from carrier animals. Imidocarb is a carbanilide derivative with anti-protozoal activity. The mode of action of imidocarb is uncertain although two mechanisms have been proposed: interference with the production and/or utilization of polyamines, or prevention of entry of inositol into the erythrocyte containing the parasite [European Agency for the Evaluation of Medicinal Products (EMEA), 2001]. The mechanism resulting in these susceptibility differences has not been described.

Our study also found that there was surprisingly more EB positive cells in cultures exposed to 4 μg/mL than cultures exposed to 1 and 2 μg/mL. The paradoxical effect of antibiotics is defined as a substantially reduced bacterial killing at antibiotic concentrations above the minimum bactericidal concentration *in vitro* (Holm *et al.*, 1991). This phenomenon was originally described for β-lactam antibiotics against gram-positive bacteria. Subsequent studies have described this phenomenon with aminoglycocides against gram-negatives and pefloxacin against *Escherichia coli* (Lorian *et al.*, 1979; Tourassowsky *et al.*, 1986). The clinical significance of the paradoxical effect is unknown. However, based on pharmacokinetic data derived from other species it would appear unlikely that imidocarb concentrations >1 μg/mL are attainable in bovine plasma without significant toxic effects (Abdullah & Baggot, 1983; Belloli *et al.*, 2002). This paradoxical effect observed *in vitro* may have little clinical relevance.

Oxytetracycline treatment was less effective at reducing EB positive cells than treatment with enrofloxacin or imidocarb. A relationship between drug concentration and percent reduction in EB positive cells was not apparent in these cultures. The greatest reduction occurred within the first 24 h of exposure to oxytetracycline. Our study revealed a statistical difference between the two isolates exposed to 8 and 16 μg/mL oxytetracycline with the VGN isolate appearing more susceptible. Tetracycline antimicrobials are considered bacteriostatic rather than bacteriocidal and the activity of these compounds is believed to be dependant on the time that drug concentrations remain above the MIC for the target organism (Scholar & Pratt, 2000). Our data suggest that oxytetracycline is rickettsiastatic and therefore, a competent host immune response would be necessary for elimination of the pathogen.

Blouin *et al.* (2002) used cultivated *A. marginale* in a cell line derived from embryos of *Ixodes scapularis* ticks to examine the effect of tetracycline on the organism. Tetracycline doses of 5, 10, 20 and 100 μg/mL resulted in significant inhibition of *A. marginale* growth as determined by ELISA. Infected cell cultures treated with medium containing 20 μg/mL tetracycline proved noninfective when inoculated into susceptible splenectomized calves. These data suggest that the MRC may be somewhere between 16 μg/mL tetracycline and 20 μg/mL tetracycline, although this data does not account for possible differences related to the culture method.

Previous studies in which successful clearance of persistent *A. marginale* infections was achieved administered oxytetracycline intravenously to cattle at 11–22 mg/kg for 5–12 days (Magonigle *et al.*, 1975; Roby *et al.*, 1978). Intramuscular oxytetracycline administered at 20 mg/kg on two, three or four occasions at intervals ranging from 3 to 7 days was also reported to be effective at eliminating carrier infections (Roby *et al.*, 1978; Kuttler *et al.*,

1980; Magonigle & Newby, 1982; Kuttler, 1983; Swift & Thomas, 1983; Rogers & Dunster, 1984). A recent study conducted by our research group demonstrated that the current recommended Office International des Epizooties (World Organization for Animal Health) treatment protocol of five injections of oxytetracycline at 22 mg/kg intravenously did not eliminate persistent OK isolate infections (Coetzee *et al.*, 2005). These conflicting reports suggest that differences in susceptibility between isolates may exist. This hypothesis is supported by the recent identification of two multidrug resistance efflux pumps in the genome of *A. marginale* (Brayton *et al.*, 2005). However, the clinical significance or activity of these pumps has yet to be examined.

The results of the present study demonstrate that short term blood culture and FACS can be used to determine the antimicrobial susceptibility of *A. marginale* and to evaluate the efficacy of novel antimicrobials *in vitro*. Cultures infected with the OK isolate exposed to 4.0 μg/mL enrofloxacin and the VGN and OK isolate exposed to 1.0 μg/mL imidocarb for 7 days were sterilized. Enrofloxacin inhibited *A. marginale* in a concentration dependent manner whereas higher concentrations of imidocarb were paradoxically less effective at reducing the number of parasitized erythrocytes. Oxytetracycline was the least efficacious antimicrobial tested and there was no relationship between oxytetracycline concentration and percent reduction in EB positive cells. With the exception of cultures exposed to enrofloxacin at 4.0 μg/mL, the isolate differences observed *in vitro* did not translate to differences in infectivity in this experiment. Further studies are required to fully elucidate the susceptibility profile of different *A. marginale* isolates. These data are essential to facilitate the development of successful chemotherapeutic protocols for the elimination of persistent *A. marginale* infections.

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