

Comparison of iatrogenic transmission of *Anaplasma marginale* in Holstein steers via needle and needle-free injection techniques

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Objective—To compare iatrogenic transmission of *Anaplasma marginale* during sham vaccination between needle and needle-free injection techniques.

Animals—26 Holstein steers confirmed negative for anaplasmosis by use of a competitive ELISA (cELISA) and an *A marginale*-specific reverse transcription (RT)-PCR assay.

Procedures—An isolate of *A marginale* was propagated to a circulating parasitemia of 2.0% in a splenectomized steer. Sham vaccination was performed in the left cervical muscles of the splenectomized parasitemic steer with a hypodermic needle fitted to a multiple-dose syringe. The same needle and syringe were used to sham vaccinate a naïve steer. This 2-step procedure was repeated until 10 naïve steers (group ND) were injected. Similarly, sham vaccination of the right cervical muscles of the splenectomized parasitemic steer and another group of 10 naïve steers (group NF) was performed by use of a needle-free injection system. Five control steers were not injected. Disease status was evaluated twice weekly for 61 days by use of light microscopy, a cELISA, and an *A marginale*-specific RT-PCR assay.

Results—Iatrogenic transmission was detected in 6 of 10 steers in group ND. Disease status did not change in the NF or control steers. Sensitivity of light microscopy, cELISA, and RT-PCR assay was 100% on days 41, 41, and 20 after sham vaccination, respectively; however, only cELISA and RT-PCR assay sustained a sensitivity of 100% thereafter.

Conclusions and Clinical Relevance—Needle-free injection was superior to needle injection for the control of iatrogenic transmission of *A marginale*. (*Am J Vet Res* 2010;71:1178–1188)

Anaplasmosis, caused by *Anaplasma marginale*, is one of the most prevalent tick-transmitted rickettsial diseases of cattle throughout the world.^{1–3} The OIE categorizes anaplasmosis as a reportable disease as a result of socioeconomic impact and international trade restrictions.⁴ However, the importance of anaplasmosis is frequently underestimated, compared with that of other diseases, because of seasonal outbreaks and disease stability in endemic areas.⁵ Clinical signs in acutely infected adult cattle include, but are not limited to, anemia, fever, icterus, and lethargy, and acute infection

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ABBREVIATIONS

16S rRNA	16S subunit of rRNA
cELISA	Competitive ELISA
CI	Confidence interval
Ct	Cycle threshold
CV	Coefficient of variation
OIE	World Organisation for Animal Health
PPE	Percentage of parasitized erythrocytes
RT	Reverse transcription

may result in death.⁶ Moreover, abortion, high mortality rates, reduced milk production, extensive treatment costs, and weight loss are key economic considerations of this disease. In 2003, it was estimated that the cost of anaplasmosis to the US cattle industry was > \$300 million/y.³

Historically, vaccination has been used to modulate disease severity. In some countries, naïve cattle are inoculated IV with bovine blood infected with *Anaplasma centrale* to reduce morbidity attributable to subsequent infection with *A marginale*.³ However, this strategy is not used or recommended in the United States because of the potential for the transfer of other blood-borne pathogens and production of erythrocytic isoantibodies.⁷ A killed

Anaplasma vaccine was previously available for use in cattle in the United States; however, it currently is available for use only in special circumstances.⁸

Cattle that develop anaplasmosis after natural infection and vaccination with live *Anaplasma* spp remain lifelong carriers despite treatment with tetracycline.^{5,9-12} Carrier cattle are responsible for horizontal, iatrogenic, and vertical transmission of anaplasmosis to naïve cattle by providing a reservoir of infective blood for biological,^{13,14} mechanical,^{14,15} and in utero infection.¹⁶⁻¹⁹

Appropriately timed application of insecticides is recommended for reducing biological transmission by hematophagous arthropods.²⁰⁻²² Because of the lack of success with treatment strategies, vaccine availability, and problematic vector control, control strategies for anaplasmosis should primarily concentrate on established methods of disease prevention.

Anaplasmosis can be transmitted during routine animal husbandry practices.^{15,22} Strategies to prevent transmission of anaplasmosis associated with vaccination have been considered impractical or uneconomical or are potentially deleterious to the success of the vaccination procedure.^{23,24} In a survey²⁵ of biosecurity protocols in veterinary practices, it was indicated that most (38/55 [69%]) veterinarians in bovine-only practices did not routinely change hypodermic needles after injection of each cow (ie, used the same needle for the injection of multiple cows).

Needle-free injection involves the use of a pneumatic-powered system to deliver vaccines, and needle-free injection techniques are efficacious for the delivery of vaccines to cattle.²⁵⁻²⁷ However, it was suggested in 1 study²⁸ that there was the potential for the transfer of blood products during the use of needle-free injectors for the administration of injections to consecutive animals. The purposes of the study reported here were to compare iatrogenic transmission of *A marginale* during simulated vaccination between needle-free and conventional needle injection techniques and to evaluate the diagnostic efficacy of light microscopy, a cELISA, and an *A marginale*-specific RT-PCR assay.

Materials and Methods

Animals—Twenty-six healthy preconditioned Holstein steers were purchased from the Kansas State University Dairy Teaching and Research Center in Manhattan, Kan. Steers were (mean \pm SD) 172.2 \pm 27.5 days old at the time of the study. All steers were screened for antibodies against *A marginale* by use of a commercially available cELISA^{4,29,30,b} and for the detection of 16S rRNA of *A marginale* by use of an *A marginale*-specific RT-PCR assay.³¹ Negative disease status was assessed through interpretation of results of the cELISA and RT-PCR assay in series.³² The study was approved by the Kansas State University Institutional Animal Care and Use and Institutional Biosafety Committees.

Randomization, housing, and husbandry—Steers were assigned to 1 of 3 treatment groups: needle injection (group ND [n = 10 steers]), needle-free injection (group NF [10]), and a noninjected control group (5). Briefly, steers were ranked in descending order on the basis of body weight, assigned a random number

by a random-number generator,^c and sorted by random number in descending order. Steers with the 5 largest random numbers were included in the noninjected control group, and the remaining 20 steers were assigned to the ND and NF groups on an alternating basis. Steers were individually housed in a biolevel 2 safety facility. An insecticide^d was applied to each steer, in accordance with the manufacturer's recommendation, at the time of entry into the biolevel 2 facility. A total mixed ration was fed at 2.5% of body weight (on an as-fed basis); the ration for each day was divided into 2 equal portions and fed twice daily. Monensin (80 g of monensin/909 kg of the total mixed ration) was the only antimicrobial included in the ration. Water was available ad libitum. When it was necessary to restrain a steer for any procedure, a rope halter was used. Steers did not receive antimicrobials that would potentially interfere with transmission of anaplasmosis for the 30 days before the study or during the study period. Furthermore, procedures that were not included in the experimental design were not performed during the study period.

Splenectomy protocol and procedure—One steer was splenectomized and used to propagate an isolate of *A marginale*. A hand-assisted, laparoscopic procedure was used for the splenectomy. Briefly, the steer was sedated by IM injection of xylazine hydrochloride (0.1 mg/kg), ketamine hydrochloride (0.1 mg/kg), and butorphanol tartrate (0.05 mg/kg). The sedated steer was restrained in right lateral recumbency. A 2% solution of lidocaine hydrochloride was infused as a local anesthetic, and a 6-cm paracostal incision was made over the anesthetized area. The incision was centered over the costochondral arch and extended approximately 3 cm caudal to the 13th rib. The abdominal cavity was entered, and the surgeon inserted a hand through this incision and bluntly dissected the connective tissue between the spleen and rumen until the splenic hilus was isolated. A 1.5-cm incision was made in the left flank, and a laparoscopic stapler, which functioned as a ligating, dividing, and stapling device, was inserted into the abdominal cavity. The surgeon used the hand that was inside the abdominal cavity to guide the laparoscopic stapler around the vascular pedicle, and 2 staples were applied around the hilus. The spleen then was dissected free from the rumen and removed from the abdomen. All incisions were closed by use of a routine 3-layer closure. Postoperative analgesia was provided via IV injection of flunixin meglumine (1 mg/kg). Penicillin G procaine (10,000 U/kg, IM, q 24 h for 3 days) was administered beginning on the day of surgery. Skin sutures were removed 14 days after surgery.

Inoculation and monitoring of splenectomized steer—A blood sample that contained a tick-transmissible Virginia isolate of *A marginale* was collected from an infected cow by a researcher at another institution,^e placed in heparin anticoagulant, and shipped on ice to our laboratory via overnight courier. This isolate was fully characterized in 1978 and was originally obtained by that researcher from the USDA Animal Parasitology Institute in Beltsville, Md.³³

Five milliliters of the heparinized blood sample was used to inoculate the splenectomized steer. The steer

was inoculated IV on day 8 after splenectomy and was then monitored daily for clinical signs of anaplasmosis, including anorexia, lethargy, and fever. Blood samples were collected daily and immediately used for determination of the PPE and PCV.

PPE determination—Blood films were made from blood samples collected into evacuated tubes containing K_2 -EDTA. An automated unit¹ was used to stain blood films with modified Wright stain.^{34,35} A Miller reticle⁸ (which has a large square with an additional square inset that is one-tenth the size of the large square) was used to determine the number of parasitized erythrocytes.³⁶ Only parasitized erythrocytes were counted in the large square; however, all erythrocytes (parasitized and nonparasitized) were counted in the smaller square. The numbers of parasitized erythrocytes and nonparasitized erythrocytes were recorded. A total of 1,000 erythrocytes were counted. The PPE was reported as a percentage and was calculated by use of the following equation, which was modified from an equation³⁶ used to measure the percentage of reticulocytes for similar conditions:

$$\text{PPE} = (\text{number of parasitized erythrocytes in the large square} / [\text{number of erythrocytes in the smaller square} \times 9]) \times 100$$

Measurement of PCV—The PCV was determined by partially filling capillary tubes³ with blood samples that were collected into evacuated tubes containing K_2 -EDTA. Tubes were centrifuged at $12,600 \times g$ for 10 minutes.³⁰ The PCV was determined by measuring¹ the height of the RBC portion and comparing it with the total height for the sample.

Experimental procedures—Transmission experiments were initiated when the splenectomized steer achieved a PPE of 2.0%. The steer was medicated by IV injection of flunixin meglumine (1 mg/kg). Fifteen minutes later, a halter was used to restrain the splenectomized parasitized steer with its head extended and tied to 1 side to provide access to the left side of its neck. Ten naïve steers (group ND) were each individually and serially restrained in a similar manner adjacent to the splenectomized parasitized steer. A multiple-dose syringe¹ was primed with a 50-mL aliquot acquired from a 1-L bag of saline (0.9% NaCl) solution.^k The syringe was fitted with a single-use, 1.7×25 -mm hypodermic needle¹ and adjusted to deliver a 2-mL injection. A sham vaccination with 2 mL of sterile saline solution was administered IM into the cervical muscles on the left side of the neck of the splenectomized parasitized steer. The same needle and syringe were then used within 60 seconds to administer a sham vaccination into the muscles on the left side of the neck of a naïve steer from group ND. This 2-step procedure was repeated until all 10 naïve steers in group ND were sham vaccinated via needle injection.

The head of the splenectomized parasitized steer then was moved to the other side to provide access to the right side of its neck. Ten naïve steers (group NF) were each individually and serially restrained in a similar manner adjacent to the splenectomized steer. A needle-free injection system^m was primed in accordance

with the manufacturer's recommendations with solution from the same 1-L bag of sterile saline solution.^k The pneumatic pressure was adjusted to 55,160 Pa for IM delivery of 2 mL of saline solution to cattle weighing < 227.3 kg. The tip of the needle-free injection apparatus was placed against the skin on the right side of the neck of the splenectomized parasitized steer. The apparatus was agitated in a circular motion to ensure there was no hair between the apparatus tip and the skin. Manual, downward pressure was used to engage and ready the apparatus for injection, and the injection trigger was depressed to release the saline solution. This process was immediately repeated on the neck of a steer in group NF. This multiple-stage procedure was repeated until all 10 naïve steers in group NF were sham vaccinated by use of the needle-free injection system.

None of the injection sites in either group was swabbed with disinfectant, and none of the needles or the needle-free apparatus tip was changed or disinfected during either injection procedure. Different persons performed all injections for the steers in groups ND and NF. Day of sham vaccination was designated as day 0.

Five control steers served as sentinel animals; furthermore, these steers were not sham vaccinated via either method (noninjected control group).

Steers were monitored daily for signs of illness for 61 days. Disease status was evaluated twice weekly (days 0, 2, 6, 9, 13, 16, 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 54, 57, and 61). On those days, 2 blood samples were collected from each steer (a 3-mL sample into evacuated tubes containing K_2 -EDTA and a 5-mL sample into evacuated tubes that contained no additive [ie, serum tubes]). Samples in the serum tubes were centrifuged at $5,000 \times g$ for 5 minutes at 4°C, and serum was harvested and stored at -80°C until subsequent analysis. Serum samples were evaluated by use of a cELISA.

cELISA—A commercial cELISA^b was used to evaluate serum samples; the cELISA was used in accordance with the method described by the OIE¹ and recommended by the manufacturer.^b The optical density of each well was measured by use of an ELISA plate reader at a wavelength of 620 nm. The percentage inhibition of each sample was calculated by use of the following equation: percentage inhibition = $100 - (\text{sample optical density} \times 100) / \text{mean optical density of a negative control sample}$. Samples with a percentage inhibition < 30% were recorded as having negative results, whereas samples with a percentage inhibition $\geq 30\%$ were recorded as having positive results.^{4,37,b}

RNA extraction and RT-PCR assay—Extraction of RNA from blood samples collected into evacuated tubes containing K_2 -EDTA was performed by use of a commercially available productⁿ; extraction was performed in accordance with the manufacturer's recommendations. Briefly, plasma was separated by centrifuging blood samples at $2,750 \times g$ for 5 minutes at 4°C. Plasma was removed with a single-use pipette. An aliquot (200 μ L) of plasma-free blood was transferred to a microcentrifuge tube. One milliliter of a monophasic solutionⁿ of guanidine thiocyanate and phenol was added to lyse the RBCs. The microcentrifuge tubes were vigorously vortexed and then allowed to sit undisturbed for

10 minutes. Chloroform (200 μ L) was added to each tube, and tubes were vigorously vortexed for 15 seconds. The solution was then allowed to sit undisturbed for 10 minutes, after which it was centrifuged at 12,000 \times g for 15 minutes at 4°C. The colorless upper aqueous phase of the solution was transferred to a new tube; 500 μ L of 2-propanol was added to each tube, and the contents were vortexed briefly. The solution then was allowed to sit undisturbed for 10 minutes, after which it was centrifuged at 12,000 \times g for 10 minutes at 4°C. Supernatant was discarded, and each pellet was washed with 1 mL of 75% ethanol; the samples were briefly vortexed, after which they were centrifuged at 12,000 \times g for 5 minutes at 4°C. Supernatant was discarded, and the remaining RNA pellet in each tube was allowed to air dry. Nuclease-free water (50 μ L) was used to resuspend each RNA pellet. Samples were stored at -80°C until analysis by use of an RT-PCR assay.

A real-time quantitative RT-PCR assay³¹ was used for the identification of 16S rRNA of *A marginale* by use of previously designed³² forward and reverse primers and a *Taq* polymerase probe designed as part of the present study. The forward and reverse primer sequences³² were 5'-CTCAGAACGAACGCTGG-3' and 5'-CATTCTAGTGGCTATCCC-3', respectively. The *A marginale* probe sequence³² was 5'-/56-FAM/CGCAGCTTGCTGCGTGTATGGT/3BHQ_1/-3'. A commercially available, 25- μ L RT-PCR assay mixture³³ that included 10 pmol of each of the forward and reverse primers, 5 pmol of each deoxynucleotide triphosphate, 187.5 nmol of MgSO₄, 11.3 pmol of *A marginale* probe, and 8 U of ribonuclease inhibitor³⁴ was placed in a tube, and 2 μ L of template was added to achieve a final volume of 25 μ L. Temperature cycles used for the RT-PCR assay were an initial complementary DNA generation cycle at 48°C for 30 minutes, then 3 minutes at 94°C, followed by 45 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 60°C for 60 seconds.

An in vitro transcript of *A marginale* plasmid DNA was prepared for use in development of the RT-PCR assay. Plasmid DNA (3 μ g) was digested with the restriction enzyme *SpeI*. The *SpeI*-digested plasmid DNA was used to generate recombinant transcripts with a T7 RNA polymerase, as described in the instructions for the kit.³⁵ Ten-fold serial dilutions (ranging from 1 billion to 1 molecule) of the in vitro transcript were analyzed in triplicate to optimize the assay in a commercially available real-time quantitative PCR system.³⁶ Real-time formation of the RT-PCR product was monitored by measuring the emitted fluorescence in the extension phase of the PCR cycles within the real-time PCR system. A reaction was assessed as positive for the template when it detected 7 fluorescent units for the emission channel of the fluorescent probe. The PCR cycle at which fluorescence was evident, which is dependent on the concentration of the template in the reaction, was regarded as the Ct value.

Linear regression was used to quantify the number of 16S rRNA template molecules in each 25- μ L reaction on the basis of the corresponding Ct value by use of the equation $y = -3.4324x + 40.38$, where y is the reported Ct value and x is the number of template molecules. The R^2 for the regression equation was 0.9973. Efficiency

of the RT-PCR assay was calculated to be 95.6%.^{30,39} Samples from a cow known to be a carrier of *A marginale* and a naïve cow were extracted and analyzed simultaneously to monitor assay performance and quality of the rRNA extraction technique.

Statistical analysis—Data were entered into a software package⁶ for subsequent calculations and manipulation. Geometric mean and CV were calculated for data acquired from recorded diagnostic test results. Diagnostic test results were also converted to a binary format (0 = negative and 1 = positive). Sensitivity and specificity with 95% CIs were calculated for light microscopy, cELISA, and RT-PCR assay for each sample ($n = 25$) at each time point.

Agreement between diagnostic results for each twice-weekly sample was assessed by calculating a κ statistic.⁴⁰ Results were compared by use of a software program⁷ in a 2 \times 2 contingency table to calculate κ via the following equations:

$$EP = ((\{a + b\}/n) \cdot (\{a + c\}/n) + (\{c + d\}/n) \cdot (\{b + d\}/n)) \text{ and } \kappa = ((\{a + d\}/n) - EP) \cdot (1 - EP)$$

where EP is the expected proportion of equal outcomes according to chance; a is the number of true-positive results, b is the number of false-positive results, n is the total number of samples, c is the number of false-negative results, d is the number of true-negative results, $(\{a + d\}/n) - EP$ is the observed proportion of equal outcomes beyond chance, and $1 - EP$ is the maximal proportion of agreement not attributable to chance. The κ statistic measures the agreement between tests on a scale from 0 to 1, with κ between 0 and < 0.4 indicating poor agreement, κ between 0.4 and \leq 0.75 indicating good agreement, and $\kappa > 0.75$ indicating excellent agreement. When κ could not be determined because of a lack of concordant results in \geq 2 of the 2 \times 2 cells, an overall proportion of agreement was calculated by dividing the sum of concordant test results by the number of samples tested.⁴⁰

The association between disease outcome and results for the 3 diagnostic testing regimens was analyzed by use of generalized linear mixed models⁶ and generalized estimating equations.³⁷ Methods were used to account for the lack of independence among repeated observations for the same animal over time. A semi-parametric survival analysis³⁸ was performed to take into account the time point at which each respective diagnostic test first detected *A marginale*. A Kaplan-Meier survival analysis³⁸ (nonparametric) was performed on the raw data to depict the amount of time elapsed after sham vaccination before a positive outcome for each of the 3 testing regimens. Values of $P < 0.05$ were used to determine significant differences.

Results

Prior to enrollment in the study, all steers were confirmed to have negative results for anaplasmosis when tested by use of the cELISA and an *A marginale*-specific RT-PCR assay. No adverse reaction attributable to surgery was detected in the splenectomized steer. The splenectomized steer was inoculated with 5 mL of

a heparinized blood sample that had a PPE of 8.1% and PCV of 30%; however, an rRNA molecule count was not determined for the inoculum because of the heparin in that blood sample.

Time elapsed from inoculation of the splenectomized steer until the development of a PPE equal to 2% was 34 days. The PPE, PCV, and number of 16S rRNA molecules were 2%, 23%, and 8.9×10^9 molecules, respectively, at that time. No adverse reactions were observed as a result of the injection methods used. In addition, the authors are not aware of any indications to suggest the use of flunixin meglumine would predispose cattle to clotting disorders, and no such complications were detected after administration of flunixin meglumine to the splenectomized parasitized steer. The splenectomized parasitized steer was euthanized 45 days after inoculation because of anorexia, lethargy, and pyrexia; however, none of the other steers were removed from the study.

At the end of the study, 6 of 10 steers in the ND group had positive results for the anaplasmosis pathogen, *A. marginale*, as determined on the basis of results of the cELISA, light microscopy, and RT-PCR assay. This represented the first, second, fourth, sixth, seventh, and tenth steers that were sham vaccinated via needle injection. All steers in the NF and control groups had negative results for anaplasmosis throughout the study. Thus, the number of steers infected with *A. marginale* differed significantly between the ND group and the NF and control groups.

The mean \pm SD predicted model-adjusted probabilities of becoming infected, comparing the ND group with the NF and control groups, were 0.60 ± 0.16 and 0.65 ± 0.18 , respectively. Because all cattle in the NF and control groups had negative results for *A. marginale*, the model-adjusted probability of a positive test result for *A. marginale* was zero for each of those groups. A steer in the ND group was significantly more likely to become infected with *A. marginale* (odds ratio, 44.6; 95% CI, 19.5 to 101.8) than was a steer in the NF or control groups.

In 25- μ L reactions with template from the 10-fold serial dilutions, the standard curve of the RT-PCR assay ranged from 100 to 1 billion molecules (Figure 1). The RT-PCR assay identified 120 molecules of 16S rRNA (Table 1) in 1 steer as early as 9 days after sham vaccination (Figure 2). Sensitivity of the RT-PCR assay was consistent in identifying steers infected with *A. marginale* in successive samples. The peak sensitivity (100%) for the RT-PCR assay was at day 20, and it was sustained through the end of the study. The peak number of 16S rRNA molecules recovered from 250 μ L of plasma-free blood was 1.6×10^6 molecules at day 41. There were no positive results for the NF and control groups by use of the RT-PCR assay.

The cELISA yielded positive results for 1 steer at 13 days after sham vaccination (Figure 2); however, sensitivity of the cELISA was inconsistent for the 4 successive samples from that steer. No infected steer was identified by use of the cELISA at day 16. A steer identified as infected with *A. marginale* at day 20 was not the same steer identified as infected at day 13. The cELISA did not have consistent sensitivity until day 34. Peak sensi-

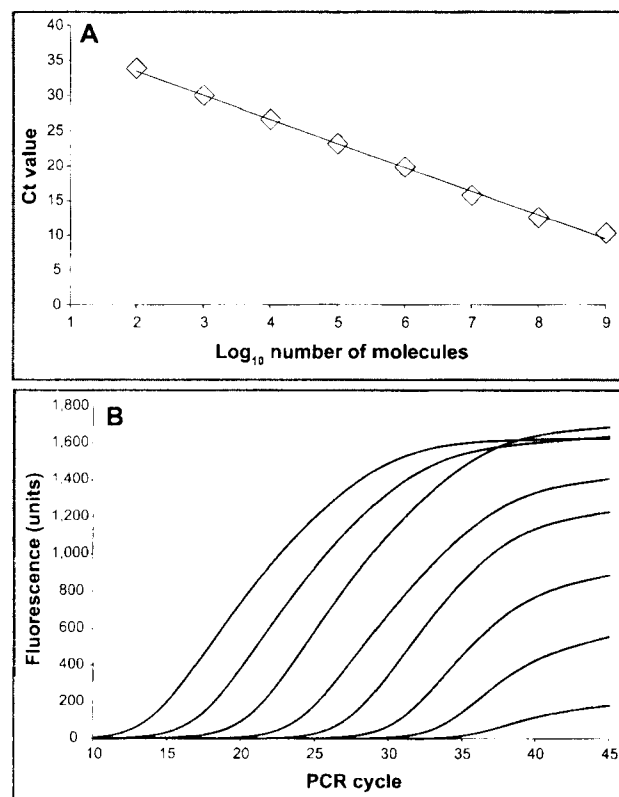


Figure 1—Sensitivity and linearity with RNA concentrations for an RT-PCR assay (A) and fluorescent emission from serial dilution templates (B). Serial 10-fold dilutions of an in vitro transcript made from *Anaplasma marginale* plasmid DNA were used. The mean Ct values from 3 separate experiments were plotted against the log₁₀ number of rRNA molecules (log₁₀ 2 and log₁₀ 9 correspond to 100 and 1 billion molecules of 16S rRNA, respectively). The equation of the line is $y = -3.4324x + 40.38$ ($R^2 = 0.9973$).

tivity (100%) for the cELISA was at day 41, and it was sustained through the remainder of the study. This coincided with the peak number of 16S rRNA molecules identified by use of the RT-PCR assay and a decreasing PCV (Table 1).

The PPE determination (use of light microscopy to examine stained blood films) detected 1 infected steer as early as 16 days after sham vaccination; however, this steer did not consistently have positive results until day 34. Interestingly, the day of peak sensitivity for light microscopy (day 41) coincided with the day of peak sensitivity for the cELISA, the peak in 16S rRNA molecules identified with the RT-PCR assay, and the decrease in PCV (Table 1). Furthermore, although they differed significantly, the odds of a positive test result for *A. marginale* by use of light microscopy were only 0.5 times as likely as the odds for a positive test result by use of the cELISA.

Variability of the performance of each diagnostic method and agreement among diagnostic methods during the peracute, acute, and chronic stages of infection were compared (Table 2). Sensitivity was inadequate among all methods during the peracute phase after sham vaccination. However, use of the RT-PCR assay, cELISA, and light microscopy identified an infected steer on days 9, 13, and 16 after sham vaccination, respectively. Peak sensitivity for the RT-PCR assay, cELISA, and light microscopy was on

Table 1—Geometric mean and geometric CV for each diagnostic test result and the PCV derived from the analysis of samples obtained from 6 steers in group ND that became infected with a Virginia isolate of *Anaplasma marginale* after sham vaccination administered by IM injection with a needle.

Day	Light microscopy*			cELISA†,§			RT-PCR			PCV		
	Mean	CV	No. of nonzero values†	Mean	CV	No. of nonzero values	Mean	CV	No. of nonzero values	Mean	CV	No. of nonzero values†
0	0	—	0	12.87	25.9	6	0	—	0	34.29	5.4	6
2	0	—	0	15.04	30.9	6	0	—	0	35.36	9.7	6
6	0	—	0	3.39	122.4	6	0	—	0	31.78	6.1	6
9	0	—	0	10.30	75.6	6	1.2×10^2	—	1	31.94	6.8	6
13	0	—	0	13.31	77.0	6	1.8×10^3	8.9	4	31.30	5.3	6
16	0.10	—	1	14.96	36.5	6	1.9×10^3	13.5	5	30.11	6.8	6
20	0	—	0	21.29	70.8	6	4.2×10^4	17.0	6	32.14	4.6	6
23	0	—	0	40.18	53.5	6	4.8×10^5	19.0	6	29.98	4.2	6
27	0	—	0	27.10	243.4	6	5.7×10^6	21.8	6	30.63	5.3	6
30	0.10	—	1	44.84	94.2	6	3.3×10^7	20.4	6	30.41	8.6	6
34	0.42	119.1	3	45.31	170.2	6	8.7×10^7	10.1	6	30.44	6.8	6
37	0.75	96.3	3	61.43	73.1	6	1.4×10^8	2.7	6	28.41	8.6	6
41	0.55	68.0	6	69.34	43.2	6	1.6×10^8	1.8	6	25.92	14.8	6
44	0.24	219.9	5	85.51	18.9	6	1.4×10^8	1.2	6	25.60	8.0	6
48	0.14	52.1	2	88.55	10.7	6	1.0×10^8	4.8	6	27.06	9.9	6
51	0.10	—	1	79.57	8.4	6	8.4×10^7	10.9	6	27.37	10.9	6
54	0.10	—	1	79.57	11.3	6	5.0×10^7	15.5	6	28.53	10.9	6
57	0	—	0	82.59	9.7	6	2.9×10^7	15.6	6	29.03	0.7	6
61	0	—	0	84.65	12.1	6	1.9×10^7	17.4	6	30.26	7.3	6

All values reported represent percentages, except for the RT-PCR assay in which the geometric mean represents the number of 16S rRNA molecules recovered from 250 μ L of plasma-free blood.

*Values reported represent the PPE. †Indicates the total number of nonzero values included in the calculation of the geometric mean and geometric CV. ‡Values reported represent the percentage inhibition. §Data were divided by 100 prior to calculating the geometric mean and geometric CV; however, the geometric mean was multiplied by 100 before it was reported. || Values reported represent the number of 16S rRNA molecules recovered from 250 μ L of plasma-free blood.

Day = Day after sham vaccination (day of sham vaccination was designated as day 0). — = Not applicable; the 95% CI was not calculated because of a lack of concordant results in ≥ 2 cells in the 2×2 contingency table.

days 20, 41, and 41, respectively. Diagnostic sensitivity was sustained throughout the remainder of the study for the cELISA and RT-PCR assay; however, sensitivity of light microscopy decreased to 0% by day 57. The RT-PCR assay was the only diagnostic method that maintained a specificity of 100% throughout the study. Poor specificity for the cELISA was caused by 8 false-positive results for steers in the control and NF groups on days 2 ($n = 1$ false-positive result), 13 (2), 16 (1), 23 (3), and 30 (1). The percentage inhibition recorded for each of these false-positive results was between 30% and 40%. The odds that an *A marginale*-infected steer would have a positive result for the RT-PCR assay was 1.34 times as likely as the odds for a positive result for the cELISA; these odds did not differ significantly ($P = 0.07$).

Agreement between results of the cELISA and RT-PCR assay as well as among results of all 3 diagnostic methods was determined. However, a proportion of concordance (0.76) was calculated on days 0, 2, and 6 after sham vaccination because of a lack of concordant results in ≥ 2 cells in the 2×2 contingency table. Sensitivity and specificity for each of these test days were 0% and 100%, respectively. The κ was calculated for the remainder of results for days 9 through 61. There was perfect agreement on day 41 when comparisons were made for 2 and 3 tests; however, excellent agreement was only sustained through the remainder of the study when results were compared for the RT-PCR assay and cELISA. At day 61, agreement between results for the cELISA and RT-PCR assay as well as among results for

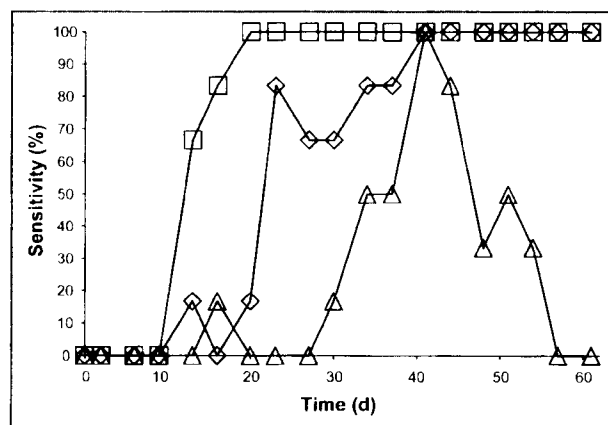


Figure 2—Sensitivity for the RT-PCR assay (white squares), cELISA (white diamonds), and light microscopy (white triangles) for detection of anaplasmosis in samples obtained from 6 steers that became infected with a Virginia isolate of *A marginale* after sham vaccination administered by IM injection with a needle. Day of sham vaccination was designated as day 0.

all 3 tests was 1.00 (95% CI, 0.72 to 1.28) and 0.75 (95% CI, 0.53 to 0.97), respectively.

Imprecision of diagnostic test results reported for the 6 steers infected with *A marginale* in the ND group was calculated as the CV of the geometric mean (Table 1). The geometric CV for the RT-PCR assay at peak sensitivity (20 days after sham vaccination) was 17%. However, this estimate did not exceed 22% throughout the study at any time point after infected steers were detected. The geometric CV for light microscopy, the cELISA, and the

Table 2—Sensitivity (Se) and specificity (Sp) for each of 3 diagnostic methods used to detect anaplasmosis in samples obtained from steers during a 61-day period after sham vaccination.

Day*	Light microscopy		cELISA		RT-PCR assay		Agreement	
	Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)	2 tests (κ)†	All 3 tests (κ)‡
9	0 (—)	100 (—)	0 (—)	100 (—)	16.7 (0–46.5)	100 (100–100)	0.12 (0–0.25)	0.08 (0–0.17)
13	0 (—)	100 (—)	16.7 (0–46.9)	89.5 (75.7–100)	66.7 (28.9–100)	100 (100–100)	0.43 (0.16–0.69)	0.31 (0.11–0.50)
16	16.7 (0–46.5)	100 (100–100)	0 (—)	94.7 (84.7–100)	83.3 (53.5–100)	100 (100–100)	0.47 (0.22–0.73)	0.4 (0.20–0.59)
20	0 (—)	100 (—)	16.7 (0–46.5)	100 (100–100)	100 (100–100)	100 (100–100)	0.68 (0.42–0.94)	0.49 (0.30–0.69)
23	0 (—)	94.7 (84.7–100)	83.3 (53.5–100)	84.2 (67.8–100)	100 (100–100)	100 (100–100)	0.79 (0.52–1.1)	0.57 (0.35–0.80)
27	0 (—)	94.7 (84.7–100)	66.7 (28.9–100)	100 (100–100)	100 (100–100)	100 (100–100)	0.88 (0.61–1.16)	0.62 (0.40–0.84)
30	16.7 (0–46.5)	94.7 (84.7–100)	66.7 (28.9–100)	94.7 (84.7–100)	100 (100–100)	100 (100–100)	0.83 (0.55–1.11)	0.64 (0.42–0.86)
34	50 (10.0–90.0)	94.7 (84.7–100)	83.3 (53.5–100)	100 (100–100)	100 (100–100)	100 (100–100)	0.94 (0.67–1.22)	0.81 (0.58–1.03)
37	50 (10.0–90.0)	94.7 (84.7–100)	83.3 (53.5–100)	100 (100–100)	100 (100–100)	100 (100–100)	0.94 (0.67–1.22)	0.81 (0.58–1.03)
41	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	1.00 (0.72–1.28)	1.00 (0.77–1.23)
44	83.3 (53.5–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	1.00 (0.72–1.28)	0.96 (0.74–1.19)
48	33.3 (0–71.1)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	1.00 (0.72–1.28)	0.84 (0.62–1.07)
51	50 (10.0–90)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	1.00 (0.72–1.28)	0.88 (0.66–1.11)
54	33.3 (0–71.1)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	1.00 (0.72–1.28)	0.84 (0.62–1.07)
57	0 (—)	100 (—)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	1.00 (0.72–1.28)	0.75 (0.53–0.97)
61	0 (—)	100 (—)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	1.00 (0.72–1.28)	0.75 (0.53–0.97)

Values in parentheses are 95% CI.
 *Values for days 0, 2, and 6 were omitted because of a lack of concordant results in ≥ 2 cells in the 2×2 contingency table. For each of these days, the calculated proportion of concordance was 0.76 (Se = 0% and Sp = 100%). †Represents agreement between results for the cELISA and RT-PCR assay. ‡Represents agreement between results for light microscopy, cELISA, and RT-PCR assay.
 See Table 1 for remainder of key.

RT-PCR assay at day 41, which corresponded to the day with the highest agreement among the 3 tests, was 68%, 43.2%, and 1.8%, respectively. The lowest estimates were at days 48, 51, and 44 for light microscopy, the cELISA, and the RT-PCR assay, respectively.

Results for the semiparametric survival analysis were expressed as hazard ratios. Hazard ratios were interpreted similarly as odds ratios, were assumed proportional over time, and were representative of the effect of a unit change in the predictor on the frequency of the outcome.⁴⁰ The hazard ratio for cattle with a positive test result for *A marginale* in the ND group by use of RT-PCR assay, compared with that for a positive test result by use of the cELISA and light microscopy, was 1.15 and 1.58, respectively. Similarly, the hazard ratio for cattle with a positive test result for *A marginale* in the ND group by use of the cELISA, compared with that for a positive test result by use of the RT-PCR assay and light microscopy, was 0.74 and 1.86, respectively. Conversely, light microscopy was the least likely to yield positive results during the study period; the hazard ratio for cattle with a positive test result for *A marginale* by use of light microscopy, compared with that for a positive test result by use of the RT-PCR assay and the cELISA, was 0.74 and 0.81, respectively.

Kaplan-Meier survival analysis was performed on raw data to compare the 3 diagnostic regimens (Figure 3). At day 0, the risk of infection for all steers exposed was 1. However, this risk decreased over time as *A marginale*-infected steers were identified.

Additionally, the semiparametric survival analysis was used to test for significant differences in the injection sequence among steers within the ND group. Once the needle was contaminated by injection of the splenectomized parasitized steer, the steers that subsequently had a positive test result for *A marginale* were the first, second, fourth, sixth, seventh, and tenth animals in the ND group. The hazard ratio for the sequence of injection (0.96) indicated that the risk of becoming infected with *A marginale* (as determined on the basis of a positive test result) was the same for all steers in the ND group. Therefore, the sequence of injection was not significantly associated with a positive test result for *A marginale*.

To further validate results for the diagnostic methods, the 15 naïve steers from the NF and control groups were each inoculated IV with 5 mL of blood obtained from 1 of the 6 ND steers iatrogenically infected with the Virginia isolate of *A marginale*. All 15 steers became infected with *A marginale* as a result of IV inoculation,

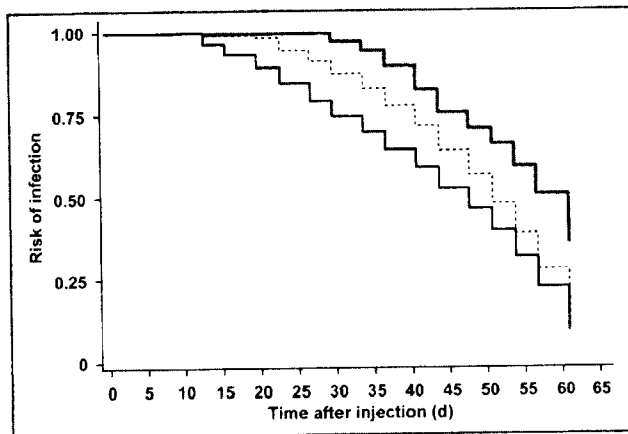


Figure 3—Kaplan-Meier survival estimate derived from the results for the RT-PCR assay (black line), cELISA (dashed line), and light microscopy (gray line) for detection of anaplasmosis in samples obtained from 6 steers that became infected with a Virginia isolate of *A. marginale* after sham vaccination administered by IM injection with a needle. Day of sham vaccination was designated as day 0.

as determined by interpretation of results of the cELISA, light microscopy, and RT-PCR assay in series. The 4 remaining steers in group ND were not challenge exposed because they were enrolled in a subsequent study.

Discussion

Anaplasmosis poses many problems to the cattle industry because of complications with disease control, eradication, and treatment. When cattle of unknown disease status are being vaccinated, it is highly recommended to use hygienic techniques. Although repetitive use of needles to inject cattle is not recommended, this practice still is in use. It may be argued that a cattle population with a stable prevalence of endemic disease may be advantageous as it results in minimizing clinical disease in adult cattle. However, this approach does not permit commingling of cattle of unknown disease status. Another disadvantage would be the transmission of other blood-borne pathogens of cattle. If the prevalence of anaplasmosis were allowed to increase, culling practices may be amplified and trade restrictions intensified between endemic and nonendemic countries because of the current use of unreliable diagnostic methods.

The study reported here was designed to evaluate the use of a needle-free injection system for the control of anaplasmosis transmission among cattle during vaccination. Needle-free injection has been validated as a tool for use in controlling horizontal transmission of *A. marginale*. Additionally, a novel RT-PCR assay was developed and evaluated for use in detecting *A. marginale* in bovine blood samples. This data set is clinically relevant because of the potential spread of *A. marginale* to naïve cattle during the performance of routine animal husbandry practices as well as for identifying deficiencies in the sensitivity and specificity of currently available diagnostic methods. To our knowledge, this is the first report in which the use of needle-free injection techniques for the control of iatrogenic transmission of anaplasmosis as well as the performance of first-, second-, and third-generation diagnostic methods at se-

quential time points after a single exposure of cattle to *A. marginale* have been evaluated.

Transmission of anaplasmosis from a known carrier to susceptible cattle via needles has been reported.¹⁵ In that report, only 1 of 5 cattle became infected. Furthermore, the authors of that report described visual detection of blood contamination on the needle between injections. In the study reported here, 6 of 10 steers became infected. Blood contamination on the needle was visible only prior to injection of the last steer in the ND group. Otherwise, the needle appeared to be safe (ie, no blood contamination) for injecting multiple cattle. Because of the random pattern of transmission and lack of significance associated with the injection sequence, the repetitive use of a needle among cattle of unknown disease status should be regarded as unacceptable. Furthermore, the fact that 6 of 10 steers exposed to an *A. marginale*-contaminated needle became infected after an IM injection leads to the hypothesis that this route of infection may be extremely common in current production systems.

Transmission of blood components during needle-free injection techniques has been reported.²⁸ This apparently was negligible for the transmission of anaplasmosis for the conditions of this study reported here. Sham-vaccination of the splenectomized parasitized steer prior to sham vaccination of each steer in the NF group robustly challenged the potential for iatrogenic transmission of a Virginia isolate of *A. marginale* via needle-free injection. However, transmission via needle-free injection techniques is not known for situations in which anaplasmosis carrier cattle may have a PPE > 2%. Similar or worse conditions are reasonably unlikely to be encountered in field settings. However, it may be necessary to account for the temporal association of previous vaccinations, disease prevalence, and timing of vaccination in regard to the seasonal distribution of clinically affected cattle when considering the use of needle-free injection techniques. Furthermore, it should be mentioned that disease resistance among breeds has not been verified.⁴¹⁻⁴³

The use of needle-free injection techniques in production settings will aid in the reduction of biohazard waste, alleviate operator injury resulting from accidental needle punctures, and eliminate the possibility of needle contamination attributable to vaccination in consumable meat products while enabling producers to maintain rates for processing of cattle that are necessary for minimizing handling and stress in the animals. Even though use of needle-free injection techniques is superior to use of needles for preventing iatrogenic transmission of *A. marginale*, it is recommended that care be used to avoid unwarranted inoculation of cattle that might result from improper removal of previously used vaccine products from the injection system because of poor cleaning techniques.²⁴ Further studies are necessary to fully evaluate the use of needle-free injection techniques for the control of other blood-borne diseases of cattle.

At the end of the study reported here, the prevalence of anaplasmosis was 24% (6/25 steers). It may be argued that the evaluation of sensitivity and specificity among the diagnostic methods included in this study

should be interpreted with caution because of the small study population and low number of infected steers; however, the accurate and precise diagnosis of anaplasmosis has historically been problematic because of diagnostic methods that lack adequate sensitivity and specificity.^{30,37,44-46} Therefore, it is important to describe the inequality of these diagnostic methods.

First-generation diagnostic methods rely on the growth or visual identification of the organism of interest. These methods have limited sensitivity and lack adequate specificity to differentiate between morphologically similar pathogens, normal intracellular structures, and stain artifacts. During the present study, light microscopy was proven to be unreliable because of false-negative results. This was attributable to the low number of circulating rickettsial organisms encountered. Even though all steers infected with *A marginale* were accurately classified at some time during the study, light microscopy had a diagnostic sensitivity of 100% only at day 41.

Second-generation methods, which rely on the identification of cell components, metabolic products, and detection of antigenic components, are currently the most commonly used techniques for disease classification in clinical medicine and research. One disadvantage of these methods is the potential for cross-reactivity among coexisting diseases. Because of similarity among MSP5 surface proteins, cross-reactivity among *A marginale*, *A centrale*, and *Anaplasma phagocytophilum* for the cELISA has been reported.^{37,46} The cELISA has had better and more sustainable sensitivity, compared with that of light microscopy. However, the cELISA did not have a sensitivity of 100% until day 41 for the ND group. A disadvantage for this technique is the cutoff value used to classify disease status. A cutoff value of $\geq 30\%$ inhibition was used in the present study to classify a steer as infected with anaplasmosis. This value led to multiple false-positive results for the NF and control groups. However, the sensitivity of this assay at the earlier time points would have been compromised if a cutoff value of 40% inhibition had been used.

Third-generation methods use nucleic acid-based techniques for the classification of disease status. These methods offer superior sensitivity and specificity over first- and second-generation methods. This study is not the first in which investigators used a nucleic acid-based technique for the diagnosis of *A marginale* in bovine blood samples.^{14,29,47-54} However, it is the first study in which a real-time quantitative RT-PCR method was used to identify 16S rRNA of *A marginale* in infected cattle. The advantages of this assay are the enhanced sensitivity for identifying rRNA targets that are present in higher quantities than a single copy of DNA per organism, the ability to quantify the genetic template, and elimination of the need for PAGE. In addition, this assay could serve as a substitute for the inoculation of splenectomized cattle with blood from cattle of unknown disease status. The major disadvantage is the cost of the reagents and equipment as well as the need for equipment that may not be readily accessible.

The RT-PCR assay had a sensitivity of 100% by day 20. This was a noticeable improvement over the use of light microscopy and cELISA for diagnosis in the

prepatent period; however, false-negative assay results were attributable to the inability of the RT-PCR assay to detect disease in 250 μ L of plasma-free blood obtained prior to day 20. Because of the performance of the diagnostic methods used during this study, the authors recommend repeated collection of blood samples from cattle of unknown disease status at 3- and 6-week intervals when the RT-PCR assay and cELISA, respectively, are used. However, light microscopy would not be recommended for determining disease in cattle with unknown disease status.

Anaplasmosis is a complex and challenging disease for stakeholders in the cattle industry, foreign policy, and research arenas. Because of the lack of substantial success with treatment strategies and problems with vaccine availability and vector control, anaplasmosis control strategies should primarily concentrate on established methods for disease prevention. This data set is clinically relevant because of the potential spread of *A marginale* to naïve cattle during performance of routine animal husbandry practices as well as the fact that it indicates deficiencies in the sensitivity and specificity of currently available diagnostic methods. Our results identified needle-free injection as a superior method for controlling the iatrogenic transmission of anaplasmosis. Furthermore, use of a novel *A marginale*-specific RT-PCR assay has the potential to impact the future of disease classification prior to local, interstate, or international movement of cattle between endemic and nonendemic countries.

- a. Anderson DE, Silveira I: Survey of large animal veterinarians' biosecurity practices (abstr), in *Proceedings*. 41st Annu Conv Am Assoc Bovine Pract 2008;244.
- b. Anaplasma antibody test kit, VMRD Inc, Pullman, Wash.
- c. Microsoft Excel 2007, Microsoft Corp, Redmond, Wash.
- d. Ultra Boss, Schering-Plough, Summit, NJ.
- e. Provided by Dr. Katherine M. Kocan, Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Okla.
- f. Hema-Tek, Ames Co, Elkhart, Ind.
- g. Miller reticle, Klarmann Rulings Inc, Litchfield, NH.
- h. Hemato-Clad plain, Drummond Scientific Co, Broomall, Pa.
- i. Critocaps microhematocrit capillary tube reader, McCormick Scientific, St Louis, Mo.
- j. Ideal Instruments Pro-Shot 50-ml. pistol grip syringe, Durvet Inc Animal Health Products, Blue Springs, Mo.
- k. B. Braun Medical Inc, Irvine, Calif.
- l. Becton, Dickinson & Co, Franklin Lakes, NJ.
- m. Pulse 250 needle-free injection system, Felton International, Lenexa, Kan.
- n. IRI Reagent, Sigma-Aldrich, St Louis, Mo.
- o. Integrated DNA Technologies Inc, Coralville, Iowa.
- p. SuperScript III reverse transcriptase, Invitrogen Corp, Carlsbad, Calif.
- q. Recombinant RNasin, Promega Corp, Madison, Wis.
- r. 17 MEGAscript high-yield transcription kit, Ambion Inc, Austin, Tex.
- s. SmartCycler II, Cepheid, Sunnyvale, Calif.
- t. WinEpiscope 2.0, CLIVE, Edinburgh, Scotland.
- u. PROC Glimmix, SAS, version 9.1, SAS Institute Inc, Cary, NC.
- v. PROC Genmod, SAS, version 9.1, SAS Institute Inc, Cary, NC.
- w. Stata, version 10.1, Stata Corp LP, College Station, Tex.

References

1. Uilenberg G. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet Parasitol* 1995;57:19-41.

2. Dumler JS, Barbet AF, Bekker CP, et al. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 2001;51:2145–2165.
3. Kocan KM, de la Fuente J, Guglielmo AA, et al. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin Microbiol Rev* 2003;16:698–712.
4. OIE. Chapter 2.4.1: bovine anaplasmosis. In: *Manual of standards for diagnostic tests and vaccines for terrestrial animals*. Paris: OIE, 2008. Available at: www.oie.int/eng/normes/mmanua/2008/pdf/2.04.01_bovine_anaplasmosis.pdf. Accessed Jul 13, 2009.
5. Rogers RJ, Shiels IA. Epidemiology and control of anaplasmosis in Australia. *J S Afr Vet Assoc* 1979;50:363–366.
6. Radostits OM, Gay CC, Blood DC, et al. Diseases caused by arthropod parasites. In: *Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses*. 9th ed. St Louis: WB Saunders Co, 2000;1401–1405.
7. Dennis RA, O'Hara PJ, Young ME, et al. Neonatal immunohemolytic anemia and icterus of calves. *J Am Vet Med Assoc* 1970;156:1861–1869.
8. Luther DG. Anaplasmosis vaccine from University Products LLC. Available at: anaplasmosisvaccine.com. Accessed May 1, 2009.
9. Lincoln SD, Eckblad WP, Magonigle RA. Bovine anaplasmosis: clinical, hematologic, and serologic manifestations in cows given a long-acting oxytetracycline formulation in the prepatent period. *Am J Vet Res* 1982;43:1360–1362.
10. Coetzee JF, Apley MD, Kocan KM. Comparison of the efficacy of enrofloxacin, imidocarb, and oxytetracycline for clearance of persistent *Anaplasma marginale* infections in cattle. *Vet Ther* 2006;7:347–360.
11. Wilson AJ, Parker R, Parker M, et al. Chemotherapy of acute bovine anaplasmosis. *Aust Vet J* 1979;55:71–73.
12. Coetzee JF, Apley MD, Kocan KM, et al. Comparison of three oxytetracycline regimes for the treatment of persistent *Anaplasma marginale* infections in beef cattle. *Vet Parasitol* 2005;127:61–73.
13. Futse JE, Ueti MW, Knowles DP Jr, et al. Transmission of *Anaplasma marginale* by *Boophilus microplus*: retention of vector competence in the absence of vector-pathogen interaction. *J Clin Microbiol* 2003;41:3829–3834.
14. Eriks IS, Palmer GH, McGuire TC, et al. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *J Clin Microbiol* 1989;27:279–284.
15. Reeves JD III, Swift BL. Iatrogenic transmission of *Anaplasma marginale* in beef cattle. *Vet Med Small Anim Clin* 1977;72:911–914.
16. Zaugg JL, Kuttler KL. Bovine anaplasmosis: in utero transmission and the immunologic significance of ingested colostral antibodies. *Am J Vet Res* 1984;45:440–443.
17. Zaugg JL. Bovine anaplasmosis: transplacental transmission as it relates to stage of gestation. *Am J Vet Res* 1985;46:570–572.
18. Potgieter FT, van Rensburg L. The persistence of colostral *Anaplasma* antibodies and incidence of in utero transmission of *Anaplasma* infections in calves under laboratory conditions. *Onderstepoort J Vet Res* 1987;54:557–560.
19. Norton JH, Parker RJ, Forbes-Faulkner JC. Neonatal anaplasmosis in a calf. *Aust Vet J* 1983;60:348.
20. Peter RJ, Van den Bossche P, Penzhorn BL, et al. Tick, fly, and mosquito control—lessons from the past, solutions for the future. *Vet Parasitol* 2005;132:205–215.
21. De Wall DT. Anaplasmosis control and diagnosis in South Africa. *Ann N Y Acad Sci* 2000;916:474–483.
22. Rodriguez-Vivas RI, Mata-Mendez Y, Perez-Gutierrez E, et al. The effect of management factors on the seroprevalence of *Anaplasma marginale* in *Bos indicus* cattle in the Mexican tropics. *Trop Anim Health Prod* 2004;36:135–143.
23. Andrews AH, Lamport A. A practical method of reducing spread of disease by hypodermic needles. *Vet Rec* 1985;116:185–186.
24. Makoschey B, Beer M. Assessment of the risk of transmission of vaccine viruses by using insufficiently cleaned injection devices. *Vet Rec* 2004;155:563–564.
25. Hollis LC, Smith JF, Johnson BJ, et al. A comparison of serological responses when modified-live infectious bovine rhinotracheitis virus vaccine and *Mannheimia haemolytica* bacterin-toxoid are administered with needle-free versus conventional needle-based injection in yearling feedlot steers. *Bovine Pract* 2005;39:106–109.
26. Huang Y, Babiuk LA, van Drunen Littel-van den Hurk S. Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic T-lymphocyte responses in mice and cattle. *J Gen Virol* 2005;86:887–898.
27. Manoj S, Griebel PJ, Babiuk LA, et al. Modulation of immune responses to bovine herpesvirus-1 in cattle by immunization with a DNA vaccine encoding glycoprotein D as a fusion protein with bovine CD154. *Immunology* 2004;112:328–338.
28. Sweat JM, Abdy M, Weniger BG, et al. Safety testing of needle free, jet injection devices to detect contamination with blood and other tissue fluids. *Ann N Y Acad Sci* 2000;916:681–682.
29. Torioni de Echaide S, Knowles DP, McGuire TC, et al. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J Clin Microbiol* 1998;36:777–782.
30. Coetzee JF, Schmidt PI, Apley MD, et al. Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers. *Am J Vet Res* 2007;68:872–878.
31. Reinbold JB, Coetzee JF, Sirigireddy KR, et al. Detection of *Anaplasma marginale* and *A. phagocytophilum* in bovine peripheral blood samples by duplex real-time reverse transcriptase PCR assay. *J Clin Microbiol* 2010;48:2424–2432.
32. Dohoo I, Martin W, Stryhn H. Screening and diagnostic tests. In: *Veterinary epidemiologic research*. Charlottetown, PE, Canada: AVC Inc, 2007;101–102.
33. de la Fuente J, Blouin EF, Kocan KM. Infection exclusion of the rickettsial pathogen *Anaplasma marginale* in the tick vector *Dermacentor variabilis*. *Clin Diagn Lab Immunol* 2003;10:182–184.
34. Teerasaksilp S, Wiwanitkit V, Lekngam P. Comparative study of blood cell staining with Wright-giemsa stain, field stain, and a new modified stain. *Lab Hematol* 2005;11:76–78.
35. Kutaish N. Automated staining of bone marrow and peripheral blood by a modified Wright's technique. *Am J Clin Pathol* 1982;77:319–320.
36. Riley RS, Ben-Ezra JM, Goel R, et al. Reticulocytes and reticulocyte enumeration. *J Clin Lab Anal* 2001;15:267–294.
37. Strik NI, Alleman AR, Barbet AF, et al. Characterization of *Anaplasma phagocytophilum* major surface protein 5 and the extent of its cross-reactivity with *A. marginale*. *Clin Vaccine Immunol* 2007;14:262–268.
38. Sirigireddy KR, Ganta RR. Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J Mol Diagn* 2005;7:308–316.
39. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* 2002;30:503–512.
40. Le CT. Probability and probability models. In: *Introductory biostatistics*. Hoboken, NJ: Wiley-Interscience, 2003;118–119.
41. Wilson AJ, Parker R, Trueman KE. Susceptibility of *Bos indicus* crossbred and *Bos taurus* cattle to *Anaplasma marginale* infection. *Trop Anim Health Prod* 1980;12:90–94.
42. Bock RE, Kingston TG, De Vos AJ. Effect of breed of cattle on innate resistance to infection with *Anaplasma marginale* transmitted by *Boophilus microplus*. *Aust Vet J* 1999;77:748–751.
43. Jonsson NN, Bock RE, Jorgensen WK. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Vet Parasitol* 2008;155:1–9.
44. Gonzalez EF, Long RI, Todorovic RA. Comparisons of the complement-fixation, indirect fluorescent antibody, and card agglutination tests for the diagnosis of bovine anaplasmosis. *Am J Vet Res* 1978;39:1538–1541.

45. Bradway DS, Torioni de Echaide S, Knowles DP, et al. Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma marginale*. *J Vet Diagn Invest* 2001;13:79–81.
46. Dreher UM, de la Fuente J, Hofmann-Lehmann R, et al. Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clin Diagn Lab Immunol* 2005;12:1177–1183.
47. Carelli G, Decaro N, Lorusso A, et al. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Vet Microbiol* 2007;124:107–114.
48. Decaro N, Carelli G, Lorusso E, et al. Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*. *J Vet Diagn Invest* 2008;20:606–611.
49. Figueroa JV, Alvarez JA, Ramos JA, et al. Bovine babesiosis and anaplasmosis follow-up on cattle relocated in an endemic area for hemoparasitic diseases. *Ann N Y Acad Sci* 1998;849:1–10.
50. Ge NL, Kocan KM, Ewing SA, et al. Use of a nonradioactive DNA probe for detection of *Anaplasma marginale* infection in field cattle: comparison with complement fixation serology and microscopic examination. *J Vet Diagn Invest* 1997;9:39–43.
51. Ge NL, Kocan KM, Murphy GL, et al. Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and in situ hybridization with a PCR-mediated digoxigenin-labeled DNA probe. *J Vet Diagn Invest* 1995;7:465–472.
52. Goff WL, Stiller D, Roeder RA, et al. Comparison of a DNA probe, complement-fixation and indirect immunofluorescence tests for diagnosing *Anaplasma marginale* in suspected carrier cattle. *Vet Microbiol* 1990;24:381–390.
53. Hoar BR, Nieto NC, Rhodes DM, et al. Evaluation of sequential coinfection with *Anaplasma phagocytophilum* and *Anaplasma marginale* in cattle. *Am J Vet Res* 2008;69:1171–1178.
54. Molad T, Mazuz ML, Fleiderovitz L, et al. Molecular and serological detection of *A centrale*- and *A marginale*-infected cattle grazing within an endemic area. *Vet Microbiol* 2006;113:55–62.