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Original Research

Prevalence and Diagnosis of Hemotrophic Mycoplasma Infection in Research Sheep and Its Effects on Hematology Variables and Erythrocyte Membrane Fragility

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Hemotrophic mycoplasma (hemoplasma) infection in research sheep can confound experimental results and contribute to morbidity and mortality. Prevalence and clinicopathologic studies historically relied on blood-smear diagnosis, but systematic studies using current molecular techniques are warranted. Here we sought to report the prevalence of subclinical infection in our study population, compare diagnostic sensitivity and specificity between blood smears and a PCR assay, and determine the effects of infection on CBC variables and erythrocyte membrane fragility. We collected whole-blood samples from 111 convenience-sampled research sheep. All samples were tested for hemoplasmas by using a PCR assay, blood smears were evaluated for visual presence of hemoplasmas, and CBC and osmotic fragility assays were performed. Subclinical prevalence, according to PCR diagnosis, was 14.1% (14 of 99) in our study population. Relative to the PCR assay, blood-smear diagnosis was 8.3% sensitive and 100% specific for hemoplasma detection. Subclinical infection was associated with changes in MCV, MCHC, RBC distribution width, and absolute monocyte count. Acute infection was associated with changes in RBC mass, Hgb concentration, MCV, MCH, MCHC, and absolute lymphocyte and monocyte counts. Acute infection was associated with increased mean erythrocyte fragility compared with that in uninfected control and treated sheep. We demonstrated that hemoplasma infection is common in our study population, blood-smear evaluation is insensitive at detecting infection, and infection is associated with changes in CBC variables and increased erythrocyte membrane fragility. These findings raise concerns regarding the suitability of hemoplasma-infected sheep for biomedical research.

Abbreviation: MEF, mean erythrocyte fragility.

Hemoplasma infection in sheep is caused by *Mycoplasma ovis* and '*Candidatus* M. haemovis.'[15,34] Recent work, however, suggests *M. ovis* and '*Ca.* Mycoplasma haemovis' represent the same organism with 2 different copies of the 16S rRNA.[11] This agent, formerly called *Eperythrozoon ovis*, is a cell-wall–free bacterial parasite that is intimately associated with the plasma membrane of sheep erythrocytes.[25] It typically is considered to be nonpathogenic in chronic infection, but it occasionally is associated with hemolytic anemia during acute infection.[24] Hemoplasma infection can confound experimental results and contribute to morbidity and mortality in research animals.[20] Infections have been reported worldwide. The clinicopathologic effects in lab animals may be a concern for biomedical research.[2]

The organism cannot be grown in culture, making its diagnosis difficult. Previous prevalence and clinicopathologic studies relied on blood-smear diagnosis or serology.[5,6,8-10,13,14,16,20,22,25-27,31] Systematic studies on the diagnosis and clinicopathologic effects of ovine hemoplasma infection using current, molecular techniques are unavailable. In addition, the contemporary prevalence of hemoplasma infection in research sheep in the United States is unknown.

The purpose of the current study was to evaluate: 1) the prevalence of subclinical hemoplasma infection in our study population of research sheep; 2) the sensitivity and specificity of blood smears to detect hemoplasma infection; 3) the effects of subclinical and acute hemoplasma infection on CBC variables; and 4) the effects of acute hemoplasma infection on erythrocyte membrane fragility. We hypothesized that: 1) subclinical hemoplasma infection is common; 2) the examination of blood smears is not as sensitive or specific as is PCR analysis for detecting hemoplasma infection; 3) subclinical and acute infection alters CBC variables; and 4) acute infection increases erythrocyte membrane fragility.

To address these questions, we collected whole-blood samples from 111 convenience-sampled research sheep as part of routine health surveillance. All samples were PCR tested by using hemoplasma-specific primers, blood smears were evaluated, and CBC analyses were performed. Osmotic fragility assays were performed on a subset of animals.

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Materials and Methods

Animals. All studies were conducted in accordance with current guidelines for animal welfare.[1,17] Animal health management and surveillance procedures are part of the animal care program administered by the Unit for Laboratory Animal Medicine. The University of Michigan University Committee on Use and Care of Animals approved the research protocols that involved sheep in these studies. All animals were housed at AAALAC-approved research facilities, and were housed in a farm setting with access to the outdoors, except when directly used for studies. Animals originated from 2 private sheep farms in southeastern Michigan or were born at the University of Michigan's Sheep Research Facility.

Hemoplasma infections occurred naturally. Infections were defined as subclinical or acute. Sheep with subclinical infection were positive for hemoplasma by PCR analysis but did not show clinical signs consistent with infection (Hct less than 20%, exercise intolerance, lethargy, and pallor). Animals with acute infection were positive for hemoplasma by PCR analysis and were reported to the veterinary staff for clinical signs consistent with hemoplasmosis.

A total of 111 sheep were sampled for these studies, comprising 99 convenience-sampled surveillance sheep; 10 lambs reported to the veterinary staff for anemia; one sheep with chronic, hemolytic anemia; and one animal that was included during follow-up for an unrelated clinical condition. There were 25 wethers and 86 ewes. The majority breed was Suffolk, but some animals were crossbreeds. Ages ranged from approximately 5 mo to 4 y. The samples were collected between December 2011 and October 2013.

Blood collection and handling. Blood samples were collected by venipuncture of cephalic or jugular sites. Blood-smear preparation, CBC analysis, and osmotic fragility assays were performed on the same day for all samples. Whole blood was stored at −80 °C until batch submission for PCR assay or was submitted fresh on the day of collection.

PCR detection for *M. ovis* and '*Ca.* Mycoplasma haemovis'. A total of 127 blood samples were tested, including 16 follow-up samples. DNA was extracted from a 200-µL aliquot of fresh or frozen whole blood from each animal by using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The manufacturer's instructions were followed, except that DNA was eluted in 75 µL (not 200 µL) of elution buffer supplied in the kit.

The hemoplasma-specific PCR assay used in the current study has been used for the detection of hemoplasma in feline, canine, bovine, and ovine species.[15,18,23,32,33] The nucleic acid sequence for the forward primer was 5′ ACG AAA GTC TGA TGG AGC AAT A 3′, and the reverse primer was 5′ ACG CCC AAT AAA TCC GRA TAA T 3′. This primer set yields amplicons of 176 bp for '*Ca.* Mycoplasma haemovis' and 193 bp for *M. ovis*. The PCR reaction mixture consisted of 2 µL DNA, 12.5 µL AmpliTaq Gold 2× PCR Master Mix (Applied Biosystems, Foster City, CA), 0.4 µM of each PCR primer (Integrated DNA Technologies, Coralville, IA), and 9.7 µL molecular-biology–grade water. The reaction conditions were: 1 cycle of 95 °C for 5 min; 45 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a final extension step of 72 °C for 5 min. The PCR products were visualized by ethidium bromide staining after electrophoresis through a 2.5% agarose gel. Figure 1 shows a representative gel image of positive and negative results.

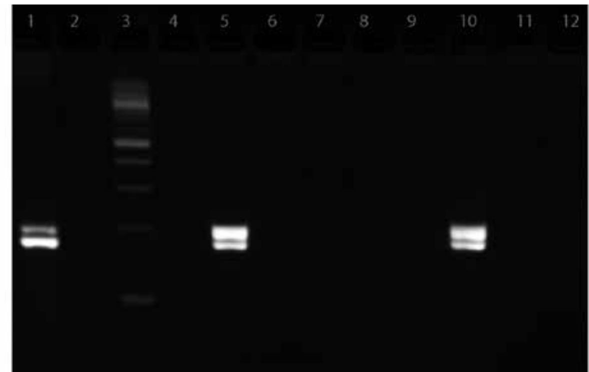

Figure 1. Representative gel image showing positive and negative PCR results. Positive controls for *M. ovis* (193 bp) and '*Candidatus* Mycoplasma haemovis' (176 bp) are in lane 1. The source of the control DNA was blood from anemic sheep. The validity of the positive control was confirmed by using nucleic acid sequence analysis. The negative control is in lane 2, and the 100-bp ladder of molecular weight markers is in lane 3. The molecular weight marker visible near the bottom of lane 3 is 100 bp, and the molecular weight maker that appears to align with the top of the top band (*M. ovis*) of the positive control is 200 bp. Lanes 4 through 12 contain PCR products from the blood of sheep tested in the current study. Lanes 5 and 10 contain PCR products from *Mycoplasma*-positive sheep that showed doublet bands.

Nucleic acid sequencing was used for confirmation of the PCR assay. Nine PCR amplicons from selected sheep ($n = 6$) were excised from gels, purified by using the QIAquick Gel Extraction Kit (Qiagen), and submitted to the Research Technology Support Facility at Michigan State University for nucleic acid sequencing. Sequences were edited by using Sequencher software (Gene Codes, Ann Arbor, MI) and analyzed by using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A subset of 10 sheep were tested in replicate by the lab of Joanne Messick (Department of Comparative Pathobiology, Purdue University College of Veterinary Medicine) for confirmation by using a published qPCR assay.[35]

Evaluation of blood smears. A total of 120 blood smears (including duplicate smears from some animals) were prepared on the same day as blood collection by using a modified Wright–Giemsa stain (Hema 3 Fixative and Solutions, Fisher Scientific, Kalamazoo, MI), per manufacturer instructions, for cytologic evaluation. Each blood smear was uniquely coded and linked to a hemoplasma PCR result. Blood smears were prepared from a total of 107 sheep that included: 95 surveillance animals, 10 lambs with acute hemoplasmosis, 1 animal with chronic hemolytic anemia, and 1 sheep that was included during follow-up for an unrelated clinical condition. Slides were evaluated by 2 independent observers (JAH and ILB) by using bright-field microscopy under oil with a 100× objective (model BX45 or BH2, Olympus, Tokyo, Japan). A minimum of 10 high-powered fields were evaluated per smear. Organisms were identified as basophilic pleomorphic coccoid or ring-form bacteria, approximately 1 to 2 µm in diameter, appearing as single organisms or short chains on the surface of erythrocytes or detached and present on the background of the slide. A hemoplasma score was assigned according to an adaptation of the following previously published scheme.[19] A slide with a score of 0 was negative for hemoplasma. Specifically, there was no

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definitive evidence of organisms in any field evaluated. Occasionally, basophilic material consistent with organisms, Howell–Jolly bodies, stain precipitate, or other debris may be seen. A score of 1 indicated a suspected positive result, for which basophilic bodies consistent with hemoplasma were found in small numbers in all fields evaluated. Because of the inherent difficulty in distinguishing organisms from stain precipitate or other artifacts, we took a conservative interpretative approach, and we considered this appearance to be suggestive, but not definitive, evidence of infection. Slides with a score of 2 were defined as positive for a light to moderate infection. On these slides, most RBC were clearly parasitized; sometimes organisms were present in the background. Slides with a score of 3 were positive for a heavy hemoplasma infection: practically all RBC were affected by numerous parasites, and organisms were readily seen in the background.

Results from the 2 observers were averaged for the final scores. The sensitivity and specificity of blood-smear evaluation were calculated, by using the hemoplasma PCR result as the true status of infection (that is, the 'gold standard'). Figure 2 shows representative blood smears for each score.

CBC analysis. Routine CBC analyses were performed on the same day as sample collection by using a hematology analyzer (Hemavet 950FS, Drew Scientific, Waterbury, CT) in the In-vivo Animal Core (Unit for Laboratory Animal Medicine, University of Michigan). Quality-assurance procedures for this laboratory consisted of daily assessment of reference samples provided by the instrument manufacturer. Quarterly assessment of values was made by comparing assay results with those of other veterinary diagnostic labs (Veterinary Laboratory Association Quality Assurance Program, Catachem, Oxford, CT). Reticulocyte variables were not included as part of the routine CBC panel.

Osmotic fragility assay. An osmotic fragility assay was adapted from a previously published procedure as an in vitro method for assessing erythrocyte membrane fragility.[30] Whole-blood samples were collected from 3 groups: 1) 12 uninfected control sheep (that is, PCR negative) from the surveillance study; 2) 9 sheep with acute hemoplasmosis (PCR positive); and 3) the same 9 sheep after treatment with long-acting oxytetracycline (20 mg/kg IM twice daily every 3 d for 4 total treatments; LA-200, 200 mg/mL, Liquamycin, Pfizer, San Francisco, CA). Osmotic fragility assays were performed on the same day as sample collection. Triplicate 20 µL K_2-EDTA-anticoagulated whole-blood samples were added to 380 µL PBS solutions of increasing osmotic pressures and incubated for 30 min at room temperature. PBS concentrations were: 0.90%, 0.80%, 0.75%, 0.70%, 0.65%, 0.60%, 0.55%, 0.50%, 0.40%, and 0.00%. Samples were centrifuged at $2000 \times g$ for 5 min at room temperature. A 100-µL aliquot of supernatant from each sample was transferred to a clear, 96-well, flat-bottom plate (Thermo Fisher Scientific, Waltham, MA). The optical density at 540 nm was measured (ELx808 Absorbance Microplate Reader, BioTek, Winooski, VT). Means of the triplicate samples were used to establish sigmoid curves with top and bottom plateaus constrained to 100% and 0%, respectively. The log IC_{50} was used as the mean erythrocyte fragility (MEF) for each sheep. Higher MEF values indicate greater erythrocyte membrane fragility.

Statistical analyses. All data were analyzed by using GraphPad Prism 6.0 (La Jolla, CA).

Descriptive statistics were calculated for CBC variables and MEF. Normality of the data distributions was assessed by using the D'Agostino and Pearson omnibus test as well as by visual inspection of the histograms. Nonnormal data were transformed and assessed for normality.

For the study of the effects of subclinical infection on routine CBC variables, data that were normally distributed were analyzed by using the unpaired 2-sided Student *t* test with Welch's correction for unequal variance. Data that failed normality were analyzed by using the Mann–Whitney test.

For the study of the effects of acute hemoplasmosis on routine CBC variables and MEF, data that were normally distributed were analyzed by using unpaired one-way ANOVA. Standard deviations were compared by using the Brown–Forsythe test. Multiple comparisons for normally distributed data were made by using the Tukey test with a single pooled variance, and *P* values were multiplicity-adjusted. Data that failed normality were analyzed by using the Kruskal–Wallis test. Multiple comparisons for nonnormal data were made by using the Dunn test, and *P* values were multiplicity-adjusted. For all tests, *P* values less than 0.05 were considered to be significant.

Results

Hemoplasma PCR assay and nucleic acid sequence analysis. Of the 127 blood samples tested in this study (of which 16 were follow-up samples), 40 were positive by the hemoplasma-specific PCR assay. The PCR assay results were validated with nucleic acid sequencing of PCR amplicons from 6 sheep and by a second PCR assay performed in another laboratory. For the 6 sheep, readable nucleic acid sequences derived from PCR amplicons ranged from 100 to 132 bp and showed 97% to 100% identity to *M. ovis* strain Michigan (GenBank accession no. CP006935) and 96% to 100% identity to comparable sequences from '*Ca.* Mycoplasma haemovis' (AB617733–AB617738). The next closest sequences, at 85% identity, were from *M. haemocervae* (KF306248–KF306250) and *M. ovis* (KF313922). For 3 of the aforementioned sheep, the PCR assay yielded doublet amplicons, which were separated by extending the time for electrophoresis (Figure 1). The derived nucleic acid sequences for the second PCR amplicon from those sheep showed 95% to 98% identity to *M. ovis* strain Michigan (CP006935) and *M. wenyonii* (GQ259756–GQ259760), *M. haemocervae* (KF306248–KF306250), and *M. ovis* (KF313922). Finding multiple distinct sequences of the 16s rRNA gene is consistent with a recent report of 2 distinct 16s rRNA genes in *M. ovis* strain Michigan.[11]

Study population prevalence of subclinical hemoplasma. Of the 99 sheep that were convenience-sampled for hemoplasma detection by PCR analysis, 14 (14.1%) were positive.

Sensitivity and specificity of blood-smear evaluation compared with PCR analysis for detection of hemoplasma infection. By using our scoring system and considering only sheep with blood-smear scores of 2 or 3 (definite positives, see Methods), the sensitivity of blood-smear evaluation for hemoplasma detection was 8.3% (3 of 36 PCR-positive cases detected) and its specificity was 100% (0 of 84 PCR-negative cases identified as positive). When sheep with blood-smear scores of 1 (suspect positives) were included, the sensitivity and specificity of blood-smear evaluation compared with PCR were 16.7% and 95.2%, respectively.

Effect of subclinical hemoplasma infection on routine CBC variables. Of the 99 sheep sampled for surveillance to investigate the effects of subclinical hemoplasma infection on routine CBC variables, 20 samples were excluded due to problems with sample handling (blood tubes were placed in direct contact with ice, caus-

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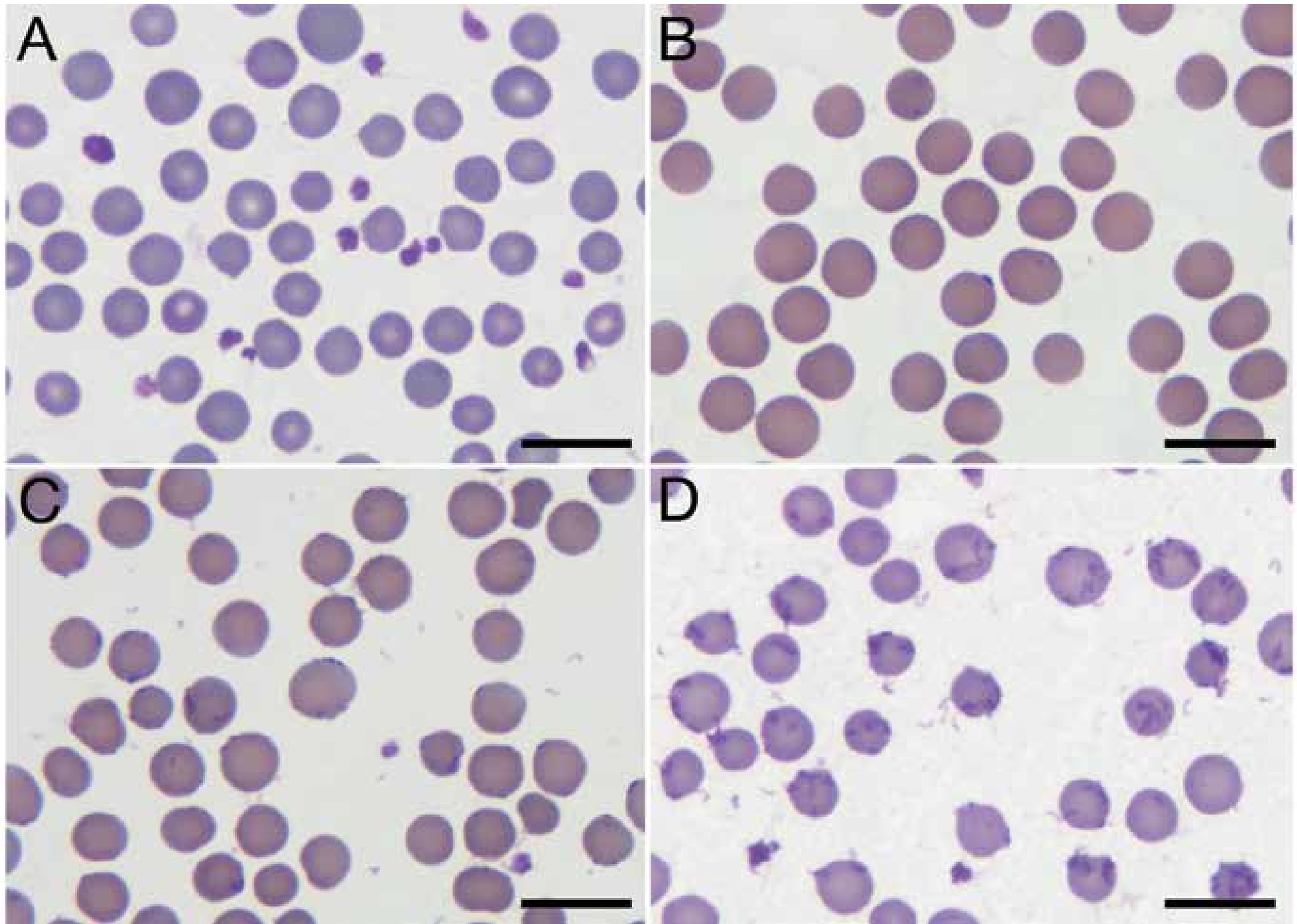

Figure 2. Hemoplasma scoring system for blood-smear evaluation. (A) Score of 0: negative. There is no definitive evidence of hemoplasma. (B) Score of 1: suspect. Basophilic bodies consistent with hemoplasma are found in small numbers. (C) Score of 2: positive, light to moderate infection. Most RBC clearly are parasitized. Organisms may occur in the background. (D) Score of 3: positive, heavy infection. Practically all RBC are affected by numerous parasites. Organisms occur frequently in the background. Modified Wright–Giemsa stain; scale bar, 10 µm.

ing gross hemolysis), leaving 79 samples for analysis. Of these 79 samples, 10 were hemoplasma-positive by PCR analysis and 69 were PCR negative. The hemoplasma PCR-positive sheep in this study were considered to be subclinically infected because none of them had overt clinical signs of hemoplasmosis (that is, Hct less than 20%, exercise intolerance, lethargy, pallor).

Table 1 describes several hematologic variables that were affected by subclinical hemoplasma infection in these surveillance animals. Compared with uninfected sheep, subclinical infection was associated with increased MCHC, RBC distribution width, and absolute monocyte count and decreased MCV.

Effect of acute hemoplasmosis on routine CBC variables. We evaluated 10 female lambs (age, 5 mo) from an unrelated study that were reported to the veterinary staff for having low Hct values (less than 20%). Three lambs had grossly hemolyzed sera, and 2 were hemoplasma-positive by blood-smear diagnosis (hemoplasma score, 2 or 3). However, 9 of the 10 lambs were hemoplasma-positive by PCR diagnosis; these 9 animals were considered acutely infected and were used to study the effects of acute hemoplasmosis and its treatment on routine CBC variables. Data from 12 uninfected (PCR-negative) animals in the surveillance study were used as controls. Data were compared between uninfected control animals to the acutely infected lambs before and after treatment.

CBC variables comparing uninfected control animals and acutely infected lambs before and after oxytetracycline treatment are described in Table 2, with ANOVA P values reported; multiplicity-adjusted P values are reported here instead. Compared with uninfected control animals, infection was associated with decreased RBC mass ($P = 0.0054$), Hgb concentration ($P = 0.0365$), and MCHC ($P < 0.0001$) and increased MCV ($P < 0.0001$), MCH ($P < 0.0001$), RBC distribution width ($P = 0.0009$), platelet mass ($P = 0.0168$), and absolute numbers of lymphocytes ($P = 0.0127$) and monocytes ($P = 0.0008$). Compared with values from infected animals before treatment, oxytetracycline treatment was associated with increased RBC mass ($P = 0.0002$), Hgb concentration ($P = 0.0001$), Hct ($P = 0.0042$), and MCHC ($P < 0.0001$) and decreased MCV ($P = 0.0154$) and MCH ($P = 0.0363$). Uninfected control and posttreatment animals differed only for Hgb concentration ($P = 0.0344$), RBC distribution width ($P < 0.0001$), and platelet mass ($P = 0.0002$).

Although treatment was associated with increased RBC mass, it did not clear infection, because 8 of 9 lambs remained PCR positive for hemoplasma after treatment.

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Table 1. CBC variables in uninfected sheep and those with subclinical hemoplasma infection

	Uninfected (n = 69)[a]	Subclinical infection (n = 10)	P
RBC (×10^6 cells/µL)	10.37 ± 1.52	10.87 ± 1.79	0.4229
Hgb (g/dL)	8.42 ± 1.29	8.84 ± 1.68	0.4631
Hct (%)	32.54 ± 4.18	31.34 ± 3.67	0.3605
MCV (fL)	**31.56 ± 2.76**	**29.15 ± 2.98**	**0.0344**
MCH (pg)	8.15 ± 0.77	8.15 ± 0.89	0.9905
MCHC (g/dL)	**25.91 ± 2.65**	**28.05 ± 2.79**	**0.0073**
RBC distribution width (%)	**19.82 ± 1.53**	**21.17 ± 1.17**	**0.0055**
Platelets (×10^3/µL)	236.7 ± 128.6	281.5 ± 204.8	0.5159
MPV (fL)	9.39 ± 1.87	8.99 ± 1.21	0.3874
WBC (×10^3 cells/µL)	7.10 ± 1.71	6.96 ± 2.65	0.8720
Neutrophil (×10^3 cells/µL)	3.28 ± 1.15	2.81 ± 1.03	0.2084
Lymphocyte (×10^3 cells/µL)	3.39 ± 0.94	3.65 ± 1.80	0.6620
Monocyte (×10^3 cells/µL)	**0.26 ± 1.77**	**0.37 ± 1.64**	**0.0445**
Eosinophil (×10^3 cells/µL)	0.17 ± 0.11	0.13 ± 0.07	0.1337
Basophil (×10^3 cells/µL)	0.01 ± 0.01	0.00 ± 0.00	0.3550

Data are shown as mean ± 1 SD. Uninfected sheep were PCR negative, whereas animals with subclinical infection were PCR positive.
Bolded values are those that differ significantly between uninfected sheep and those with subclinical hemoplasma infection.
[a]n = 67 for MPV.

Table 2. CBC variables in uninfected controls and acutely infected sheep (before and after treatment)

	Uninfected (n = 12)	Infected, before treatment (n = 9)	Infected, after treatment (n = 9)	ANOVA P
RBC (×10^6 cells/µL)	9.90 ± 0.68	6.89 ± 1.32	10.56 ± 0.92	0.0002
Hgb (g/dL)	8.07 ± 0.69	6.99 ± 1.03	9.16 ± 1.11	0.0002
Hct (%)	33.06 ± 2.79	30.52 ± 4.31	36.81 ± 4.35	0.0056
MCV (fL)	33.36 ± 1.03	44.73 ± 4.38	34.83 ± 2.25	0.0001
MCH (pg)	8.15 ± 0.26	10.21 ± 0.81	8.66 ± 0.55	0.0001
MCHC (g/dL)	24.41 ± 0.56	22.88 ± 0.79	24.87 ± 0.75	<0.0001
RBC distribution width (%)	18.58 ± 0.73	22.67 ± 2.18	24.61 ± 3.39	<0.0001[a]
Platelet (10^3/µL)	174.8 ± 113.2	374.4 ± 225.0	494.4 ± 103.1	0.0002[a]
MPV (fL)	9.13 ± 1.54	10.23 ± 1.91	8.97 ± 0.63	0.1497
WBC (×10^3 cells/µL)	7.58 ± 1.61	8.68 ± 3.37	7.79 ± 1.83	0.5467
Neutrophils (×10^3 cells/µL)	3.68 ± 0.99	2.57 ± 1.09	3.33 ± 1.16	0.0789
Lymphocytes (×10^3 cells/µL)	3.42 ± 0.89	5.41 ± 2.18	3.90 ± 1.20	0.0148[a]
Monocytes (×10^3 cells/µL)	0.26 ± 0.11	0.53 ± 0.19	0.37 ± 0.14	0.0012
Eosinophils (×10^3 cells/µL)	0.21 ± 0.16	0.16 ± 0.16	0.18 ± 0.11	0.4891
Basophils (×10^3 cells/µL)	0.02 ± 0.05	0.02 ± 0.03	0.00 ± 0.01	0.2339

Data are shown as mean ± 1 SD. Uninfected control sheep were PCR negative, whereas infected animals were PCR positive. Samples were collected from treated animals 3 d after the final oxytetracycline dose.
[a]Brown-Forsythe $P < 0.05$

Effect of acute hemoplasmosis on MEF. MEF data comparing uninfected control sheep with infected animals both before and after treatment are described in Figure 3. Infected sheep had higher mean pretreatment MEF values compared to posttreatment (P = 0.0136) and uninfected animals (P = 0.0001). Mean MEF values did not differ between uninfected and posttreatment sheep (P = 0.2411). These findings demonstrate the efficacy of treatment at correcting, at least temporarily, the increased erythrocyte fragility noted before treatment.

Discussion

In these studies, we evaluated: (1) the prevalence of subclinical hemoplasma infection in our study population of research sheep; (2) the sensitivity and specificity of blood-smear evaluation for the detection of hemoplasma infection; (3) the effects of subclinical and acute hemoplasma infection on CBC variables; and (4) the effects of acute hemoplasma infection on erythrocyte membrane fragility. Our results show that (1) subclinical hemoplasma infection is common in our sheep; (2) blood-smear evaluation is

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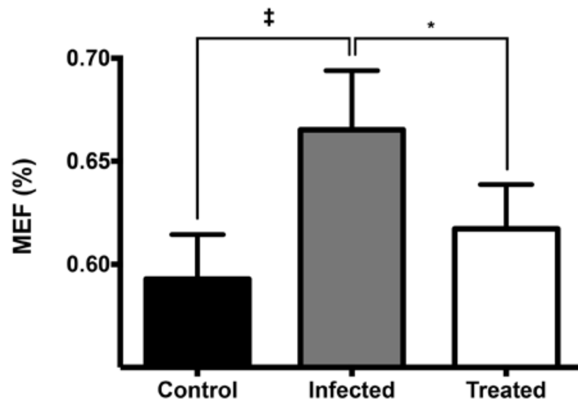

Figure 3. Mean erythrocyte fragility (MEF) values of uninfected control and acutely infected sheep (before and after treatment). As the test solution becomes more hypotonic, erythrocytes lyse; erythrocyte fragility is proportional to MEF. Mean MEF values were: control, 0.5928%; infected, 0.6652%; and treated, 0.6172%. The multiplicity-adjusted *P* values were: control compared with infected (‡, $P = 0.0001$); control compared with treated ($P = 0.2411$), and infected compared with treated (*, $P = 0.0136$). Error bars are the 95% confidence intervals of the means.

insensitive at detecting hemoplasma infection; (3) subclinical and acute hemoplasma infections are associated with changes in CBC variables; and (4) MEF is increased in acutely infected sheep.

Our first finding—that subclinical hemoplasma infection is common in our study population—is supported by the results of the surveillance study, in which 14.1% (14 of 99) convenience-sampled sheep without clinical signs of hemoplasmosis tested positive for the organism by PCR analysis. Our second finding—that blood-smear evaluation lacks sensitivity in detecting hemoplasma infection—is supported by the observations of 2 independent and experienced clinicians (JAH and ILB) using a carefully designed scoring system adapted from the literature.[19] Only 8.3% of PCR-positive slides were definitively identified (hemoplasma score of 2 or 3) by blood smear. However, 100% PCR-negative slides were correctly identified, leading to a higher specificity than we expected. Our third finding—that subclinical and acute hemoplasmosis is associated with changes in CBC variables—is supported by the CBC studies. Subclinical infection was associated with changes in MCV, MCHC, RBC distribution width, and absolute monocyte count. Acute infection was associated with changes in these variables and in RBC mass, Hgb concentration, MCH, platelet mass, and absolute lymphocyte count. CBC changes were relatively large in acute infection. Finally, our fourth finding—that acute hemoplasmosis increases erythrocyte membrane fragility—is supported by our observations that the mean MEF values of infected sheep were higher than those of uninfected and treated animals.

The 14.1% PCR prevalence of subclinical hemoplasma infection in our convenience sampled study population is lower than what was reported from other studies that used PCR for diagnosis. In Japan, 8 of the 33 sheep (24%) tested in a previous study were positive for hemoplasma by PCR analysis.[34] These animals came from 3 sources, and it is unclear if any had clinical signs at the time of sampling.[34] In Hungary, 17 of 33 sheep (52%) tested were positive for hemoplasma by PCR assay, and a subset of these animals had clinical signs at the time of sampling.[15] Reported prevalences may differ because of differences in sampling criteria, PCR methods, geography, breed, age, sex, or husbandry practices. The biggest limitation of our prevalence study is that we used convenience sampling; it is unlikely that our results reflect the true prevalence of subclinical hemoplasma infection, and they should not be generalized beyond this population. Nevertheless, these data suggest that hemoplasmosis cannot be considered an uncommon infection, and its true prevalence may vary by institution.

The CBC changes we saw during acute hemoplasma infection are consistent with findings in the literature. In the most comprehensive study on the effects of experimental infection on CBC variables, diagnosis was based on blood-smear evaluation after experimental inoculation with hemoplasma-infected blood.[27] As in the cited study,[27] we similarly found decreases in RBC mass, PCV, Hgb concentration, and MCHC and increases in MCV, MCH, and the absolute monocyte count. However, the increase we saw in absolute lymphocytes was not seen previously. Other studies of naturally and experimentally infected animals report similar changes in a limited number of variables, including RBC mass, PCV, and Hgb concentration.[5,6,8,9,13,14,25,31] These studies relied on blood smears for diagnosis and statistical methods were not described.

Our mean MEF results are consistent with several reports in the literature. In 2 previous studies, healthy sheep had mean MEF values of 0.53% ($n = 9$) and 0.58% ($n = 11$).[4,7] Our results regarding erythrocyte fragility are similar to those of a study involving experimentally infected sheep, which reported a mean MEF value of 0.65% in control animals ($n = 9$) and 0.72% in infected animals ($n = 7$).[27] Although the author of that study[27] did not report any statistical analyses, when we used his data to compare uninfected and infected animals, the mean MEF values were different ($P = 0.0002$, unpaired student's *t* test).

One of the shortcomings of our CBC and MEF studies was that our control group was older than were the infected lambs; some of the observed differences may have been age-related. Despite this factor, the treatment-associated increases in RBC mass, Hgb concentration, and Hct and decreases in MCV and MEF strongly support that these effects were related to hemoplasma infection. Another limitation of the study was that the lambs with acute infection were sampled only once after treatment, so we could not determine whether the CBC and MEF changes were transient or persistent. Therefore, the chronicity of these changes should be investigated in future studies.

Acute hemoplasmosis is one cause of hemolytic anemia in sheep, and other differential diagnoses should be considered when evaluating a clinical case. Other differential diagnoses include copper toxicity, ingestion of *Brassica* plants, leptospirosis, clostridial toxicosis caused by *C. perfringens* type A or *C. haemolyticum*, and infection by *Babesia* spp.[12] Other common causes of anemia include trauma, external or internal parasitism, and chronic inflammation. Parasitism (*Haemonchus contortus*) and trauma were ruled out as causes of anemia in the acutely infected lambs; other causes were unlikely in light of the husbandry and management practices used. According to the clinical presentations, leptospirosis and clostridial toxicosis were unlikely bacterial causes of anemia in these lambs, so the changes seen after treatment (improved RBC indices, decreased hemoplasma score, no gross serum hemolysis) suggest that hemoplasma was the causative agent.

The selection of healthy animals in biomedical research is crucial to avoid false results, and hemoplasma infection in research

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animals has the potential to confound experiments. CBC changes in subclinical and acute infections may confound studies relying on hematologic variables. In addition, subclinical infections may become clinically relevant as a result of experimental stress, manipulation, or concurrent immunosuppressive condition. For example, hemoplasma-associated hemoglobinuria developed after splenectomy of 2 infected sheep.[29] Blood-smear–detectable parasitemia became evident after the injection of betamethasone (1 mg/kg once daily for 5 d) in 3 experimentally infected animals.[13] In a natural setting, a ewe developed severe clinical signs of hemoplasmosis associated with lambing.[28] Studies that carry an inherent risk of hemolysis may be particularly vulnerable to the confounding effects of hemoplasma infection, given that the risk of hemolysis may increase with increasing MEF.[3,21] Although we showed that mean MEF values did not differ between uninfected controls and treated sheep, we also demonstrated that the infection was not cleared; therefore the recurrence of clinical symptoms is a possibility. Given these possibilities, screening research animals by PCR analysis may be advisable to avoid hemoplasma-related complications.

To identify hemoplasma-infected animals, PCR analysis is preferred over blood-smear evaluation, because the sensitivity of blood-smear evaluation is relatively poor. In addition, PCR assays can be used as a confirmatory test for animals with clinical signs consistent with hemoplasmosis or for those with evidence of organisms on blood smear (hemoplasma score 1 to 3). It should be noted that PCR or culture assays designed for nonhemotrophic *Mycoplasmas* (for example, *M. bovis, M. ovipneumoniae*) will not detect hemotrophic *Mycoplasmas*. Care should be taken that the assay used is specific for hemoplasmas, particularly when samples are sent out for analysis. Hemoplasma-sensitive qPCR protocols have been reported in the literature.[35]

In summary, we demonstrated that hemoplasma infection is common in our study population of research sheep and that blood-smear evaluation is insensitive at detecting infection. Both subclinical and acute infections are associated with changes in the CBC and MEF; some of these alterations resolve after treatment, although the infection is not cleared. PCR screening for hemoplasma is advisable in sheep intended for biomedical research use.

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