Detection of hemoplasma infection of goats by use of a quantitative polymerase chain reaction assay and risk factor analysis for infection

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OBJECTIVE
To develop and validate a real-time quantitative PCR (qPCR) assay for the detection and quantification of Mycoplasma ovis in goats and investigate the prevalence and risk factors for hemoplasma infection of goats located in Indiana.

ANIMALS
362 adult female goats on 61 farms.

PROCEDURES
Primers were designed for amplification of a fragment of the dnaK gene of M. ovis by use of a qPCR assay. Blood samples were collected into EDTA-containing tubes for use in total DNA extraction, blood film evaluation, and determination of PCV. Limit of detection, intra-assay variability, interassay variability, and specificity of the assay were determined.

RESULTS
Reaction efficiency of the qPCR assay was 94.45% ($R^2$, 0.99; slope, –3.4623), and the assay consistently detected as few as 10 copies of plasmid/reaction. Prevalence of infection in goats on the basis of results for the qPCR assay was 18.0% (95% confidence interval, 14% to 22%), with infected goats ranging from 1 to 14 years old, whereby 61% (95% confidence interval, 47% to 73%) of the farms had at least 1 infected goat. Bacterial load in goats infected with M. ovis ranged from $1.05 \times 10^3$ target copies/mL of blood to $1.85 \times 10^5$ target copies/mL of blood; however, no bacteria were observed on blood films. Production use of a goat was the only risk factor significantly associated with hemoplasma infection.

CONCLUSIONS AND CLINICAL RELEVANCE
The qPCR assay was more sensitive for detecting hemoplasma infection than was evaluation of a blood film, and production use of a goat was a risk factor for infection. (Am J Vet Res 2016;77:882–889)

Hematropic Mycoplasma spp (hemoplasmas) are epierythrocytic bacteria lacking cell walls that have been found in several mammalian species. The bacteria were previously known as Hemobartonella spp and Eperythrozoon spp, but studies conducted by use of the 16S rRNA gene sequences led to reclassification of these bacteria as members of the Mycoplasma genus. Mycoplasma ovis and Candidatus Mycoplasma hemovis have been found to infect sheep and goats, whereas M. ovis–like organisms have been identified in white-tailed deer (Odocoileus virginianus), reindeer (Rangifer tarandus), South American deer (Blas-tocerus dichotomus, Mazama nana, and Mazama americana), and Japanese serows (Capricornis crispus). Comparison of the 16S rRNA gene sequences of M. ovis and Candidatus Mycoplasma hemovis has revealed several nucleotide mismatches and a 17-bp deletion in the sequence of Candidatus Mycoplasma hemovis, which results in 97% identity between these 2 organisms. However, recent evidence suggests that rather than 2 genetically distinct species, a single species infects sheep; this single species has 2 dissimilar copies of the 16S rRNA gene in its genome. Information regarding hemoplasma infections in goats is limited, and the authors are aware of no published reports of hemoplasma infections of goats in the United States. On the other hand, sheep infected with M. ovis were first described in South Africa in 1934 and have subsequently been found throughout the world. Prevalence of hemoplasma infections in Japan and Hungary ranged from 44% to 67%, as determined on the basis of results for a qPCR assay and in China from 26% to 41%, as determined on the basis of results for a conventional PCR assay. Molecular evidence for infection with Candidatus Mycoplasma hemovis in sheep in Japan and Hungary and goats in Hungary has been reported.

ABBREVIATIONS
CI Confidence interval
Cq Quantification cycle
GAPDH Glyceraldehyde 3-phosphate dehydrogenase

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Transmission of infection by several blood-feeding arthropods\textsuperscript{14,15,a} as well as by blood-contaminated equipment has been reported. Clinical signs associated with hemoplasma infections in sheep differ depending on whether the infection is acute or chronic. Acute infections are characterized by hemolytic anemia\textsuperscript{12} and decreased exercise tolerance.\textsuperscript{16} Chronic hemoplasma infections are characterized by a reduction in body weight and ill thrift and are believed to be the main source of infection within a herd. Stress, illness, pregnancy, or immunosuppression can cause reversion of a chronic infection to an acute infection.\textsuperscript{17}

Before molecular assays (specifically PCR assays) were available, examination of Giemsa-stained blood films and serologic assays were used to diagnose hemoplasma infections. However, sensitivity and specificity of blood film evaluation are low, and seroconversion indicates only exposure and not necessarily active infection. It is also known that bacterial concentrations in the blood fluctuate during the course of a hemoplasma infection; at their nadir, the concentrations may not be detected by use of microscopy. This is especially true for animals that are chronically infected. Furthermore, it has been reported that the degree of bacteremia in goats detected by use of microscopy\textsuperscript{18} is lower than that in sheep; this finding has been substantiated with molecular techniques.\textsuperscript{15}

The use of more sensitive and specific molecular techniques, such as conventional PCR assays and qPCR assays, has greatly improved the ability to diagnose hemoplasma infection in various host species. However, PCR and serological assays have been used to investigate risk factors for hemoplasma infection in dogs, cats, and sheep.\textsuperscript{19–22}

The use of molecular diagnostic methods (specifically qPCR assays) allows for the detection and quantification of hemoplasma infections.\textsuperscript{23} Advantages of the use of a nucleic acid dye in a qPCR assay are that it is inexpensive and easy to use and binds to any double-stranded DNA; thus, there is no requirement for design and optimization of probes. Melting curve analysis of the qPCR products also can be used to determine whether single specific products are amplified; thus, nonspecific product formation can be distinguished by use of a melting curve profile of the amplified target DNA.\textsuperscript{25,24} However, differentiation of melting curves in a hemoplasma coinfected sample requires that the size and nucleotide sequences selected by the primers differ between the hemoplasma species.\textsuperscript{25}

It is estimated that there are 41,700 goats and 55,000 sheep in Indiana\textsuperscript{26}; however, the prevalence of hemotropic \textit{Mycoplasma} spp–infected goats and sheep in Indiana is unknown. Therefore, the objectives of the study reported here were to develop and validate a qPCR assay for the detection and quantification of \textit{Movic}\textsuperscript{27} in goats, evaluate the prevalence of hemoplasma infection in goats located in Indiana, and determine risk factors for hemoplasma infection in infected goats.

### Materials and Methods

#### Animals

Blood samples were collected via jugular venipuncture from 362 female goats located on 61 farms in Indiana. Samples were collected between June 2012 and June 2013 and were placed in tubes containing EDTA as an anticoagulant. The goats were involved in an unrelated study\textsuperscript{27} designed to estimate the prevalence of infection with \textit{Coxiella burnetii} in goats in Indiana. Inclusion criteria were that does be >1 year old and not pregnant at the time of sample collection. Each producer selected up to 10 eligible does/farm to be used for sample collection. Each producer provided informed consent for use of the does in the study. All procedures involving goats were approved by the Animal Care and Use Committee at Purdue University.

Most of the farms were located in rural and suburban settings and consisted of goats raised for noncommercial purposes, as determined by one of the authors (AEB). Herd management practices differed greatly among farms, with most goats having substantial access to the outdoors.

#### Cyt measurement and blood film preparation

Measurement of PCV and total solids concentration and evaluation of blood films were performed on all EDTA-anticoagulated blood samples. A plain microhematocrit capillary tube was used to obtain an anticoagulated blood sample from the blood collection tube; microhematocrit capillary tubes were centrifuged for 5 minutes at 12,100 X g. Blood films were prepared by use of EDTA-anticoagulated blood stained with modified Wright stain, and the presence or absence of hemoplasmas was assessed microscopically.

#### DNA extraction

Genomic DNA was extracted from 100 µL of EDTA-anticoagulated goat blood with a commercially available kit\textsuperscript{9} used in accordance with the manufacturer's instructions. Extracted DNA was stored at -20°C. Nuclease-free water was extracted as a negative control sample.

#### Housekeeping gene for the qPCR assay

A housekeeping gene (GAPDH) was used to confirm the presence of amplifiable DNA from all samples. Each sample was assayed in a single well. Forward (5'-GGG TCA TCA TCT CGC CTA-3') and reverse (5'-GGT CAT AAG TCC CTC CAC GA-3') primers were designed\textsuperscript{4} to amplify a 176-bp sequence of the GAPDH gene of goats. The PCR mixture contained 12.5 µL of a mastermix,\textsuperscript{4} 0.75 µL of each primer (300nM), 6.0 µL of DNase-free water, and 5.0 µL of template DNA. Cycling conditions consisted of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Melting curve analysis was performed on
the PCR product by use of a step-up cycling process from 60° to 95°C.

**Development of a standard curve**

A forward (5-ATT GAA TCA GGT CAG CCA AA-3) and reverse (5-TCC ATC AGA AAC ATC AAG CA-3) primer were designed on the basis of a fragment of the dnaK gene of *M. ovipneumoniae* strain Michigan (GenBank accession No. CP006935.1). These primers were used to amplify a 498-bp fragment of the gene in a blood sample obtained from an *M. ovipneumoniae*-infected sheep. The PCR product was amplified with a conventional PCR assay and purified from an agarose gel by use of a commercially available kit. Cloning of the products was conducted in accordance with the manufacturer’s protocols by use of a commercial vector system. Plasmids were extracted from transformed strain JM109 cells by use of a commercially available kit. A recombinant plasmid containing the fragment of the dnaK gene of *M. ovipneumoniae* (which was confirmed by Sanger sequencing) was used as a template for creating standard curves for quantification of hemoplasma DNA in unknown samples. Concentration of plasmid containing the dnaK insert was 49 ng/µL as assessed by use of a spectrophotometer. The number of copies of the recombinant plasmid was determined through the use of an online calculator. Values used included the following: amount of DNA = 49 ng and length of template = 3,015-bp vector + 498-bp insert = 3,513 bp, which resulted in 1.29 X 10^10 copies. Subsequently, 10-fold serial dilutions of plasmid DNA (10^9 copies of plasmid/reaction to 1 copy of plasmid/reaction) were prepared by adding 15.5 µL of recombinant plasmid DNA to 184.5 µL of 1X tris-EDTA buffer combined with herring sperm DNA (50 µg/mL). The standard curve was created by plotting the logarithm of the initial copy number of input plasmid DNA against the C_q value.

**Primers for real-time PCR assay for detection of *M. ovipneumoniae***

Forward and reverse primers were designed on the basis of a fragment of the dnaK gene of the published *M. ovipneumoniae* strain Michigan genome by use of primer design software. The forward primer was dnaK F (5-TGC TTT GCT AAT GTA GGT TCC-3), and the reverse primer was dnaK R (5-TTG TTG GAG AAA GCG CTA AGA-3). Use of these primers resulted in a 194-bp product.

Optimal primer concentrations were determined by testing combinations of forward and reverse primers in triplicate at 200, 300, 500, and 700nM by use of plasmids containing a fragment of the dnaK gene of *M. ovipneumoniae*. The lowest concentration of a primer that did not cause dimer formation and had a low C_q value was selected. The reaction mixture consisted of 12.5 µL of mastermix, 200 to 700nM forward and reverse primers, 5.0 µL of template DNA, and sufficient DNase-free water to achieve a final volume of 25 µL. The PCR protocol used was the same as that used previously for the GAPDH gene. To determine the optimum annealing temperature of the primers dnaK F and dnaK R, the qPCR assay was performed over a range of temperatures (57° to 62°C) by use of duplicates at each of several plasmid copy numbers (1 X 10^9, 1 X 10^9, 1 X 10^5, 1 X 10^5, and 1 X 10^5).

**qPCR assay**

A thermocycler was used for absolute quantification assays with plasmid DNA serial dilutions included in each run. A no-template sample that used DNase-free water was included in each reaction as a negative control sample, whereas DNA from the blood of an infected sheep (confirmed by qPCR assay and DNA sequencing) was used as a positive control sample. The reaction was performed in a final volume of 25 µL, and samples were assayed in duplicate. The PCR mixture contained 12.5 µL of a commercially available mastermix, 0.5 µL of each primer (200nM), 6.5 µL of DNase-free water, and 5.0 µL of template DNA. Cycling conditions consisted of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 61°C for 1 minute. Melting curve analysis was performed on the PCR product by use of a step-up cycling process from 61° to 95°C.

**Validation of the qPCR assay**

Efficiency of the primers for DNA detection or limit of detection was determined by use of standard curves generated from 10-fold dilutions of plasmids containing the dnaK gene insert. Interassay variability was assessed by assaying triplicates of all concentrations of the plasmid dilution on 5 days. Intra-assay variability was performed on a single qPCR assay with 5 replicates of each of the plasmid dilutions. Analyses of the results included determination of the coefficient of variation, mean coefficient of variation, and amplification efficiency. The DNA extracted from mycoplasmas known to infect goats or sheep (*Mycoplasma mycoides* subsp *capri*, *Mycoplasma arginini*, *Mycoplasma agalactiae*, *Mycoplasma ovipneumoniae*, *Mycoplasma capricolum* subsp *capripneumoniae*, and *Mycoplasma mycoides* subsp *mycoides*) and other bacteria that may be associated with septicemia in these ruminant species (*Borrelia burgdorferi*, *Pasteurella multocida*, *Escherichia coli*, *Clostridium perfringens*, *Clostridium difficile*, *Listeria monocytogenes*, *Listeria ivanovii*, *Yersinia pseudotuberculosis*, *Mannheimia haemolytica*, *Leptospira interrogans* serovar pomona, *L. interrogans* serovar icterohemorrhagiae, and *Salmonella enterica* serovar Typhimurium) was used to determine specificity of the qPCR assay. Additionally, DNA extracted from hemotropic mycoplasmas (*Candidatus Mycoplasma turicensis*, *Mycoplasma beneficialis*, *Candidatus Mycoplasma hemominutum*, *Mycoplasma parvum*, *Mycoplasma suis*, *Mycoplasma hemocanis*, and a hemoplasma from a deer) was also included. Bacterial pathogens were grown in solid or liquid media, and DNA was extracted by use of a commercially available kit. Conventional PCR assays with universal eubacterial primers described elsewhere were also included.
were used to confirm successful DNA extraction from the bacteria.

**DNA sequencing**

To confirm the target size and specificity of the products, DNA sequencing of amplicons from a subset (approx 15%) of field samples was performed; $C_q$ values for the samples ranged from 29.2 to 34.0. The qPCR amplicons were separated by electrophoresis on a 3% agarose gel and were subsequently stained with ethidium bromide. A commercially available kit was used for purification of DNA from the gel. The fragment of the dnaK gene was directly sequenced in both directions by personnel at the Purdue Genomics Core Facility using the previously described qPCR primers dnaK F and dnaK R.

**Statistical analysis**

Variables assessed as risk factors for hemoplasma infection in the goats included age, herd size, production use, farm location, lactation status, and the presence of sheep on a farm. Mixed logistic regression, with farm as a random effect, was used for the analysis of risk factors on both farm and goat levels. Age, herd size, production use of a goat, and farm location were assigned to categorical groups prior to analysis. For statistical comparisons, 3 age groups were created: < 2 years old (n = 106), 2 to < 8 years old (252), and ≥ 8 years old (22). Three groups were created for herd size: < 14 goats (n = 104), 14 to < 37 goats (151), and ≥ 37 goats (96). Two categories of production use were created: dairy (n = 267) and meat (94). Three regions for farm location in Indiana were created: Northern (n = 96), Central (160), and Southern (106). The Fisher exact test was used to assess whether anemia status was associated with a positive result for the qPCR assay. The Spearman rank correlation coefficient was used to assess the association between PCV and hemoplasma load in blood. All analyses were performed with commercially available statistical software.m,n The 95% CI was calculated for observed prevalence. Values of $P < 0.05$ were considered significant. Samples that yielded indeterminate results for the qPCR assay were categorized as negative for analysis purposes.

**Results**

**Goats**

Blood samples were obtained from 362 female goats located on 61 farms in Indiana for use in a qPCR assay. Goats ranged from 1 to 14 years of age (median, 2 years); the age of 2 goats was not reported. Herd size ranged from 2 to 150 goats (median, 20 goats); herd data were not available for 11 goats. There were 267 goats used for dairy production and 94 used for meat production; production use for 1 goat was not reported.

**Validation of the qPCR assay**

Concentration for the forward and reverse primers was 200nM. Reaction efficiency of the qPCR assay ranged from 90.1% to 98.5% during testing with annealing temperatures of 57° to 62°C; therefore, the assay was subsequently performed at an annealing temperature of 61°C. Reaction efficiency of the qPCR assay was 94.4% ($R^2 = 0.99$; slope = $-3.4623$; Figure 1). Results for the qPCR assay were linear over 8 orders of magnitude; the qPCR assay detected 1 copy of plasmid/reaction in 45% of the assays. Thus, the limit of detection by use of the plasmid dilutions was 10 copies/reaction, which resulted in consistent detection in 100% of the assays. Intra-assay and interassay repeatability of the qPCR assay were determined (Table 1). The $C_q$ value used for the cutoff for samples with positive results was 34.0. This value was determined by calculating the mean of the mean $C_q$ values for $10^1$ and 1 copy of plasmid/reaction from the interassay data. Samples from goats infected with $M$ ovis were used to assess the ability of the qPCR assay to detect replicates with $C_q$ values < 34.0 and ≥ 34.0. Four to 6 replicates of each of 5 samples with a $C_q$ value < 34.0 were tested. The assay detected $M$ ovis in 93.3% of the replicates. However, when 6 replicates of each of 3 samples with a $C_q$ value > 34.0 were tested, detection of $M$ ovis decreased to 72.2%.

![Figure 1](image)

$y = -3.4623x + 34.991$

$R^2 = 0.99$

**Table 1**—Intra-assay and interassay repeatability of a qPCR assay developed for the detection of Mycoplasma ovis infection in goats.

<table>
<thead>
<tr>
<th>Plasmid copies</th>
<th>Intra-assay repeatability*</th>
<th>Interassay repeatability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^1$</td>
<td>5.42 ± 0.35 6.47</td>
<td>5.81 ± 0.15 2.60</td>
</tr>
<tr>
<td>$10^2$</td>
<td>7.18 ± 0.05 0.67</td>
<td>7.37 ± 0.12 1.62</td>
</tr>
<tr>
<td>$10^3$</td>
<td>10.58 ± 0.06 0.58</td>
<td>10.89 ± 0.12 1.11</td>
</tr>
<tr>
<td>$10^4$</td>
<td>14.22 ± 0.07 0.49</td>
<td>14.55 ± 0.14 0.96</td>
</tr>
<tr>
<td>$10^5$</td>
<td>17.54 ± 0.13 0.72</td>
<td>18.02 ± 0.11 0.64</td>
</tr>
<tr>
<td>$10^6$</td>
<td>21.99 ± 1.67 7.55</td>
<td>21.89 ± 0.16 0.74</td>
</tr>
<tr>
<td>$10^7$</td>
<td>24.34 ± 0.08 0.33</td>
<td>24.89 ± 0.16 0.66</td>
</tr>
<tr>
<td>$10^8$</td>
<td>28.25 ± 0.32 1.16</td>
<td>28.76 ± 0.23 0.81</td>
</tr>
<tr>
<td>$10^9$</td>
<td>31.37 ± 0.63 2.01</td>
<td>32.21 ± 0.46 1.44</td>
</tr>
<tr>
<td>$10^{10}$</td>
<td>34.81 ± 0.80 2.29</td>
<td>35.94 ± 1.56 4.36</td>
</tr>
</tbody>
</table>

*Plasmid was diluted in 1X tris-EDTA buffer combined with herring sperm DNA (30 µg/mL). †Value reported is mean ± SD. CV = Coefficient of variation.
Specificity of the qPCR assay

There was no amplification of DNA from any of the bacterial pathogens tested by use of the primers developed on the basis of a fragment of the dnaK gene of *M. ovis*. When the universal eubacterial primers were used, a band with the expected size was present for all of the tested bacteria, which indicated successful DNA extraction.

Results for the qPCR assay and microscopy

There was amplification of the targeted sequence for the housekeeping gene GAPDH for all DNA samples, which indicated successful DNA extraction. Mean melting temperature for the GAPDH amplicons was 82.6°C. Of the 362 samples, 65 (17.9%; 95% CI, 14.1% to 21.3%) had positive results for *M. ovis* when tested by use of the qPCR assay. Mean C<sub>q</sub> was 32.1 (range, 26.4 to 34.0), and mean melting temperature was 74.6°C. Bacterial loads in goats infected with *M. ovis* ranged from 1.05 X 10<sup>3</sup> target copies/mL of blood to 1.85 X 10<sup>4</sup> target copies/mL of blood (mean, 1.31 X 10<sup>3</sup> target copies/mL of blood). An additional 60 samples with C<sub>q</sub> values > 34.0 (range, 34.1 to 39.9; mean, 35.5) and mean melting temperature of 74.7°C were categorized as indeterminate. No bacteria were observed on blood films of samples collected from any of the goats.

DNA sequencing

Sequencing of amplicons of 11 of 65 (16.9%) samples that yielded positive results by use of the qPCR assay were confirmed to be *M. ovis*. These amplicons had 98% to 100% identity with the dnaK gene of the *M. ovis* strain.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2 vs 2 to &lt; 8</td>
<td>0.96</td>
<td>0.49–1.87</td>
<td>0.993</td>
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<tr>
<td>&lt; 2 vs ≥ 8</td>
<td>1.01</td>
<td>0.33–3.04</td>
<td></td>
</tr>
<tr>
<td>2 to &lt; 8 vs ≥ 8</td>
<td>1.05</td>
<td>0.34–3.19</td>
<td></td>
</tr>
<tr>
<td>Herd size (No. of goats)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 14 vs 14 to &lt; 37</td>
<td>0.89</td>
<td>0.42–1.89</td>
<td>0.727</td>
</tr>
<tr>
<td>&lt; 14 vs ≥ 37</td>
<td>1.23</td>
<td>0.52–2.89</td>
<td></td>
</tr>
<tr>
<td>14 to 37 vs ≥ 37</td>
<td>1.38</td>
<td>0.60–3.18</td>
<td></td>
</tr>
<tr>
<td>Farm location in Indiana</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North vs Central</td>
<td>1.35</td>
<td>0.63–2.91</td>
<td>0.707</td>
</tr>
<tr>
<td>North vs South</td>
<td>1.32</td>
<td>0.52–3.33</td>
<td></td>
</tr>
<tr>
<td>Central vs South</td>
<td>0.97</td>
<td>0.44–2.16</td>
<td></td>
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<tr>
<td>Lactation status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes vs no</td>
<td>1.5</td>
<td>0.71–3.37</td>
<td>0.273</td>
</tr>
<tr>
<td>Presence of sheep on farm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes vs no</td>
<td>0.51</td>
<td>0.18–1.41</td>
<td>0.187</td>
</tr>
<tr>
<td>Production use</td>
<td></td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Dairy vs meat</td>
<td>3.3</td>
<td>1.49–7.31</td>
<td></td>
</tr>
</tbody>
</table>

*Values were considered significant at P < 0.05.

Prevalence of infection and risk factors for infection

Prevalence of hemoplasma infection in goats, as determined by use of the qPCR assay, was 18% (95% CI, 14% to 22%), and 61% (95% CI, 47% to 73%) of farms had at least 1 hemoplasma-infected goat. Goats used for dairy purposes had a significantly (P = 0.004) higher prevalence of hemoplasma infection (3.3-fold increase; 95% CI, 1.5- to 7.3-fold), compared with the prevalence in goats used for meat (Table 2). Frequency of hemoplasma infection did not differ significantly among age groups; it was 17% (95% CI, 10% to 25%) for the youngest age group, 18% (95% CI, 14% to 24%) for the middle age group, and 14% (95% CI, 3% to 35%) for the oldest age group. Frequency of hemoplasma infection did not differ significantly among herd sizes; it was 18% (95% CI, 11% to 27%) for the smallest herds, 19% (95% CI, 13% to 26%) for the intermediate-sized herds, and 15% (95% CI, 8% to 23%) for the largest herds. No significant correlation (ρ = 0.045; P = 0.727) was found between PCV and hemoplasma blood load for 64 samples with positive results for the qPCR assay. Analysis of anemia status and positive results for the qPCR assay revealed no significant (P = 0.515) correlation. Mean ± SD PCV for goats with positive and negative results for the qPCR assay was 26.9 ± 4.6% and 27.2 ± 5.2%, respectively.

Discussion

To the authors’ knowledge, the study reported here was the first in which hemoplasma infection in goats in the United States has been described. In the present study, development and validation of a qPCR assay for the detection of *M. ovis* in the blood of goats were described, and risk factors for infection were analyzed. Results indicated that the qPCR assay had high sensitivity for detection of *M. ovis* in goat blood samples. Ten copies of plasmid/reaction were consistently detected. The positive cutoff was set at a C<sub>q</sub> value of 34.0 to minimize the amount of indeterminate results. However, because DNA sequencing of amplicons from samples with indeterminate results was not performed, it is difficult to know whether the positive cutoff value was selected correctly.

Reports about the prevalence of *M. ovis* infection in goats determined by use of molecular methods are scarce. In the study reported here, prevalence was 18% (65/362), which is similar to results in a study<sup>13</sup> of the prevalence in goats in Hungary (20% [4/20]).<sup>13</sup> Goats in that study, similar to goats in the present study, were in good physical condition and were not anemic. A study<sup>11</sup> in which investigators used a semi-nested PCR assay to detect *M. ovis* in goats in China revealed a prevalence of 41% (151/372), but use of a PCR assay on the same samples yielded a prevalence of only 26% (97/371). Although prevalence of *M. ovis* infection in sheep in the United States, as determined on the basis of PCR assays, is largely unknown, a prevalence of 14% for subclinical hemoplasma infec-
tion of sheep in Michigan was reported in a recent study.\textsuperscript{36} In sheep in Japan with no apparent signs of infection, prevalence was 24\% (as determined by use of a PCR assay).\textsuperscript{12} On the other hand, 67\% and 55\% of sheep with clinical signs in Hungary\textsuperscript{2} and Japan\textsuperscript{10} respectively, were infected with \textit{M ovis}, as determined on the basis of testing with a qPCR assay.

Information is limited regarding the herd prevalence for \textit{M ovis} in goats. A herd prevalence of 61\% (37/61) in the present study was, to the authors' knowledge, the first estimated herd prevalence determined by use of an appropriate sample size. Investigators of the study\textsuperscript{13} of goats collected samples from 20 of 310 goats in only 2 herds (150 goats of one herd and 160 goats of the other herd); therefore, an appropriate comparison of herd prevalence estimates cannot be made.

Several hematophagous vectors have been implicated in the transmission of \textit{M ovis}\textsuperscript{14,15,16}; however, their role in the natural spread of infection has not been established. It is believed that the successful transfer of erythrocytes infected with \textit{M ovis} requires high amounts of biting activity by vectors when circulating bacterial loads are low.\textsuperscript{31} However, when bacterial loads are high, such as during an acute infection, biting activity of vectors can be low. Investigators of previous studies\textsuperscript{10,12} on hemoplasma infection in goats and sheep have reported lower numbers of circulating bacteria as well as lower antibody titers and a lack of clinical signs in goats, compared with results for sheep. Bacterial loads of 4 goats from the 150-goat herd in the study\textsuperscript{13} in Hungary ranged from 62 to 1,160 copy numbers/\(\mu\)L of blood (mean, 470.5 copy numbers/\(\mu\)L of blood). In the study reported here, mean bacterial load for 65 goats was 13.1 copy numbers/\(\mu\)L of blood (range, 1 to 185 copy numbers/\(\mu\)L of blood). Goats from the 150-goat herd of the Hungarian study as well as from the present study were in good physical condition. The low copy numbers per microliter of blood detected in goats of both studies, in addition to the lack of clinical signs, suggested chronic infection.

Not surprisingly, microscopy in the study reported here was found to be insensitive for detecting hemoplasma infections in goats with low bacteremia. A study\textsuperscript{8} that involved 10 research sheep (1 was anemic and 9 were healthy) determined that half the sheep had positive results for \textit{M ovis} infection when tested by use of a qPCR assay; however, no bacteria were detected by use of light microscopy for the 9 healthy sheep. Four healthy sheep had bacterial loads that ranged from 1.3 to 760 copies/\(\mu\)L, which suggested chronic infection, whereas the anemic sheep had 10\(^{10}\) copies/\(\mu\)L, which was consistent with an acute infection.\textsuperscript{8} The ability of a qPCR assay to detect and quantify low-level hemoplasma infections in the absence of clinical signs may be particularly useful in epidemiological studies and for reducing the rate of false-negative results.

Although the pathogenicity of \textit{M ovis} in goats is unknown, no correlation between \textit{M ovis} blood loads and PCV, nor between anemia and positive results for the qPCR assay, was detected in the present study. It is possible that there was too few samples with positive results (n = 65) to enable us to detect a correlation, or perhaps antimicrobials were administered to some animals, which could have caused the hemoplasma blood load to be below the detection limit of the assay. However, studies of alpacas confirmed to be chronic carriers of \textit{Candidatus Mycoplasma haemolamae} did not detect a significant association between bacterial load and clinical signs or anemia,\textsuperscript{33} and no correlation was detected between PCV and positive results for a qPCR assay.\textsuperscript{34} In general, chronic hemoplasma infection in an otherwise healthy animal does not lead to overt disease and anemia. Nevertheless, more subtle changes, such as production-related issues (growth rate and weight gain, susceptibility to other infections, and reproduction issues) and modulation of innate and adaptive immune responses, need to be further investigated in animals with chronic infection.

Investigators of other studies conducted to analyze risk factors for hemoplasma infection in cows and sheep have reported that farm location\textsuperscript{22-25} and living conditions and age\textsuperscript{35} were significantly different between infected and noninfected animals, and only the production use of a goat (dairy vs meat) was found to have a significant effect on infection status in the present study. Dairy goats had a 3.3-fold increase in hemoplasma infection, compared with that of meat goats (Table 2). Although it was not significant, lactating goats had a 2.5-fold increase in hemoplasma infection, compared with that of nonlactating goats. The small sample size, in particular the low number of meat goats that had positive results and that were lactating, limited our ability to test for differences in effects of lactation and positive results for the qPCR assay between meat and dairy goats. It is possible the immunosuppressive effects during birthing and the continued care of a kid (or kids) could cause a recrudescence of bacterialia in these animals during stressful periods. There is a decrease in immune function during the periparturient period in sheep.\textsuperscript{36,37} However, a larger sample size consisting of lactating and nonlactating goats would be needed to further investigate this relationship.

A limitation of the present study was that information collected through questionnaires was geared toward identifying risk factors for \textit{C burnetii} infection. Thus, specific questions pertinent to the transmission of hemoplasmas (eg, exposure to blood-sucking parasites, population density of the herd [population per unit area], and detailed housing aspects) were not posed. Also, the study reported here involved the use of convenience-based sampling (does at least 1 year old and not pregnant were inclusion criteria), so the estimated prevalence obtained may not have been representative of goat herds in Indiana.
The presence of sheep on goat farms was found to have a protective effect against a positive result for the qPCR assay in samples obtained from goats, although the finding was not significant (Table 2). This outcome raised questions as to whether confounding variables were influencing the relationship between the presence of sheep on goat farms and goats having a positive result for the qPCR assay. Overall, the small sample size, in particular the low number of goats with positive results for the qPCR assay, limited our ability to test for interactions among risk factors of interest.

In the present study, a qPCR assay was developed; the assay was sensitive and specific for the detection and quantification of M. ovis in infected goats. This assay may be especially useful in epidemiological studies to further characterize subclinical hemoplasma infection in goats.

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Footnotes

References


24. O’Mahony J, Hill C. A real time PCR assay for the detection and quantitation of Mycobacterium avium subsp. paratuberculosis.


