

Molecular prevalence of *Anaplasma marginale* and associated risk factors in beef cattle herds from Ohio: a cross-sectional study

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OBJECTIVE

To estimate the prevalence of *Anaplasma marginale*, the causative agent of bovine anaplasmosis, in beef herds from Ohio; evaluate farm identity and animal age as risk factors; and examine serologic cross-reactivity with *Anaplasma phagocytophilum*, an emerging disease agent.

ANIMALS

4 beef cattle herds (n = 327) sampled between December 2020 and December 2021.

PROCEDURES

To address the broader investigation of characterizing *Anaplasma* spp and genotypes in Ohio, herds with a history of clinical anaplasmosis were targeted. Blood was screened for antibodies to *Anaplasma* spp using a competitive enzyme-linked immunosorbent assay with seropositive samples tested for *A marginale* using real-time PCR. If negative, samples were also tested for *A phagocytophilum*.

RESULTS

We estimated a statewide molecular prevalence of 38.53% (95% CI, 33.26% to 43.81%), with some farms exhibiting higher prevalence than others (19.40% to 56.86%). Accounting for farm identity, the odds of an animal becoming infected increased by 1.41 (95% CI, 1.28 to 1.58) for every year in age. Forty-four animals tested seropositive but PCR negative for *A marginale*. Out of these, 2 tested positive for *A phagocytophilum*.

CLINICAL RELEVANCE

Our study was the first to report prevalence estimates for bovine anaplasmosis in Ohio. Although prevalence was higher than other states, this is most likely due to our sampling approach. Our results suggested that older animals are more likely to be infected with *A marginale*, and when animals are instead infected with *A phagocytophilum*, serology alone can be misleading wherever the 2 species co-occur. Our study can guide wider epidemiological studies for informing management in Ohio.

Bovine anaplasmosis is caused by the intraerythrocytic bacterium *Anaplasma marginale* and is one of the most widespread infectious diseases of cattle.¹ Infection can lead to anemia, icterus, jaundice, reduced milk production, abortion, and death in adult cattle, while calves are more likely to be asymptomatic.¹ In recovered cattle, infection with *A marginale* results in persistent infections, allowing individuals to serve as chronic carriers.² Given the illness and economic losses that follow bovine anaplasmosis,^{1,2} characterizing its prevalence is of paramount importance for informing prevention and control efforts.

In the US, bovine anaplasmosis is endemic, with genotypes grouped into 2 regional groups: south-eastern and west-central.¹ *A marginale* can be transmitted via biological (tick vectors), mechanical (fomites and biting insects), and transplacental routes (cow to calf). However, some genotypes of *A marginale* can be transmitted only by ticks, such as *Dermacentor* spp, whereas others only via mechanical means.¹ Although wild ruminants can serve as reservoirs for *A marginale*, the evidence for their implication is weak, suggesting that persistently infected cattle are likely the primary reservoir for ana-

plasmosis.^{1,3} This is especially important to consider because current recommendations for antimicrobial treatment may not eradicate infection, allowing for cattle to persist as chronic carriers.⁴⁻⁶ Therefore, identifying carriers can help reduce disease transmission via surveillance-based interventions.

Although several studies have been done in various regions within the US to document the prevalence of anaplasmosis in beef cattle,⁷⁻¹⁴ none have been reported from Ohio, a state with a significant number of cattle herds that support a robust agricultural economy.¹⁵ Therefore, our objective with this study was to characterize statewide and within-herd prevalence of *A marginale* in beef cattle herds from Ohio and investigate how farm identity and animal age influenced infection status.

To accomplish our objective, we first screened cattle for antibodies to *Anaplasma* spp using serology and then tested seropositive samples for *A marginale* using molecular techniques. However, when cattle are instead infected with the related *Anaplasma phagocytophilum*, seropositivity can ensue due to immunological cross-reactivity.^{1,16-18} Historically, this has not been a significant concern because of the restricted range of the blacklegged tick (*Ixodes scapularis*), *A phagocytophilum*'s primary vector.² However, due to ongoing tick range expansion,¹⁹ *A phagocytophilum* is now an emerging disease agent in Ohio.²⁰ Therefore, we also tested seropositive but *A marginale* PCR-negative samples for *A phagocytophilum* to examine serologic cross-reactivity, a diagnostic issue that may become more commonplace as the blacklegged tick expands its range.

The study herein was part of a broader investigation about the characterization of *Anaplasma* spp and genotypes circulating within beef herds in Ohio. However, in this paper we focus solely on the prevalence of bovine anaplasmosis for 4 farms, the effects of farm identity and animal age on infection status, and serologic cross-reactivity with *A phagocytophilum*. Molecular characterization will be presented elsewhere.

Materials and Methods

Animal sampling

Between 2020 and 2021, we opportunistically sampled 4 beef cattle herds located at farms in the following counties of Ohio: Clinton, Lorain, Jackson, and Coshocton. In 2021, the total cattle inventory in Ohio was estimated to be 1,260,000, with 5,800 located in Clinton county, 11,000 in Lorain county, 10,600 in Jackson county, and 24,000 in Coshocton county (**Figure 1**).²¹

We sampled each herd on separate days, as follows: Lorain on December 16 and 17, 2020; Clinton on January 26, 2021; Jackson on June 24, 2021; and Coshocton on November 19 and December 21, 2021. These herds were intentionally selected because they had a clinical history of anaplasmosis. Although we sampled the entire Clinton and Jackson herds, we sampled approximately 77% and 93% of the Coshoc-

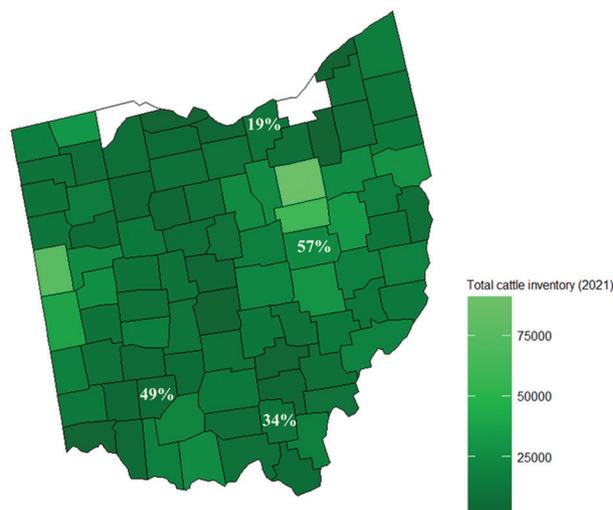


Figure 1—Map of the total cattle inventory estimates for 2021 according to the USDA for each county in Ohio. Two counties shown in white had no available estimates. Approximate estimates of molecular prevalence for 4 beef herds sampled in this study are shown for the counties of Lorain, Clinton, Coshocton, and Jackson.

ton and Lorain herds, respectively (**Table 1**), because some animals were either too young or exceptionally difficult to safely restrain. While our sampling approach restricted extrapolation of our results to other farms in Ohio, it allowed us to address our broader investigation mentioned previously.

For each animal, we collected data on sex and age. Age was determined on the basis of written farm records or verbal communication with farmers. Unfortunately, despite several attempts to reach the owner, we were unable to retrieve cattle age data for the Lorain herd. We noted that although 29 cattle from the Coshocton herd had been treated with oxytetracycline at least once in October 2021, none of the other herds were treated.

Blood collection

Up to 10 mL of blood was collected from the jugular or coccygeal vein by use of a sterile needle (Vacutainer single-sample 18-gauge needle; Becton, Dickinson and Co) for each animal. We separated blood into 2 collection tubes: one tube (Vacutainer 4-ml serum separator; Becton, Dickinson and Co) for serology and another tube (Vacutainer 4-ml EDTA; Becton, Dickinson and Co) for DNA extraction and PCR, ensuring sufficient volumes for completing the intended assays. EDTA tubes were immediately frozen on dry ice before transport to a -20 °C freezer, whereas serology tubes were held at ambient temperature until processing or processed on-site. We noted that we could collect blood for only PCR and not for serology from 3 cattle at the Coshocton farm. All procedures involving live animals were approved by The Ohio State University IACUC (protocol No. 2020A00000105).

Serology

Blood samples were centrifuged for 15 minutes at 1,600 X g (Sprint 6H; Benchmark Scientific Inc),

Table 1—Descriptive statistics for 4 beef herds (individual and combined) in Ohio. Seropositive blood samples were tested with real-time PCR for *Anaplasma marginale* to estimate the prevalence of bovine anaplasmosis.

Farm county	Herd size (n)	No. tested (%)	Median age (IQR) [†]	Raw % PCR positive (No. positive/No. tested)	Predicted % PCR positive [‡] (No. positive/No. tested)
Clinton	85	85 (100%)	5 (4.5)	49.41% (42/85)	45.88% (39/85)
Coshocton	66	51 (77.27%)	3 (5.5)	56.86% (29/51)	52.94% (27/51)
Jackson	124	124 (100%)	3.5 (5.0)	33.87% (42/124)	23.39% (29/124)
Lorain	72	67 (93.06%)	—	19.40% (13/67)	—
All	347	327 (94.24%)	4 (5.0)	38.53% (126/327)	36.54% (95/260)

[†]Age measured in years. [‡]As a function of farm identity and cattle age.

with serum decanted and frozen at -20°C . Serum was subsequently tested for antibodies to *Anaplasma* spp by use of an *Anaplasma* antibody test kit (VMRD Inc). Positive and negative controls were run in duplicate and triplicate, respectively, and only samples with $\geq 30\%$ inhibition were considered positive, per manufacturer instructions. This test is a competitive enzyme-linked immunosorbent assay that detects antibodies to MSP5, a surface protein common among closely related *Anaplasma* spp.¹

Molecular detection

For seropositive samples, we extracted DNA from EDTA blood samples using the PureLink Genomic DNA mini kit (Thermo Fisher Scientific), following the manufacturer instructions for blood. We tested DNA extracts in probe-based real-time PCR assays using published primers and probes for *A marginale*²² and *A phagocytophilum*.²³ Thermocycling conditions were 95°C for 3 minutes, 50 cycles of 95°C for 3 seconds followed by 60°C for 45 seconds. Samples were considered positive when the cycle threshold value was ≤ 40 with a characteristic curve. All PCR reactions included molecular-grade water as a negative control and a synthetic gBlock gene fragment (Integrated DNA Technologies) of *A marginale* (AY841153.1) or *A phagocytophilum* (AY151054.1) as positive controls.

Statistical analysis

We generated descriptive statistics and conducted data analyses using R in RStudio (version 1.4.1103; The R Project for Statistical Computing). For spatial visualization, we used the R package *usmap*, and to calculate 95% CI for prevalence, we used function *BinomCI* in the R package *DescTools* using the Wald method.

A generalized linear model with a logit link and binomial SE distribution was used to test whether farm identity alone influenced infection status with *A marginale*. Significance was examined with χ^2 tests. Post hoc Tukey multiple comparisons with *P* adjustment (single-step method) were performed using the R package *multcomp*.²⁴ To analyze the additive effect of age on infection status with *A marginale*, while accounting for farm identity, we also used a generalized linear model with a logit link and binomial SE distribution (Lorain was excluded because no age data were available). Statistical significance was set at $\alpha = 0.05$.

Results

We sampled 327 beef cattle from 4 farms in Ohio, with most ($n = 124$) of them in the Jackson farm and the remaining in the Clinton (85), Lorain (67), and Coshocton (51) farms. The median age was 4 years (range, 0.5 to 20 years), and the majority were females ($n = 311$).

We tested 324 samples for the presence of *Anaplasma* spp antibodies and found 169 tested positive with an apparent prevalence of 52.16% (95% CI, 46.72% to 57.60%). If we assumed an ELISA sensitivity of 100% and specificity of 99.7%,²⁵ the true prevalence would be similar at 52.02%. In addition to the seropositive samples, we also tested the 3 samples of the cattle from the Coshocton farm using real-time PCR. Out of the samples tested, 126 were positive on real-time PCR, thereby estimating an apparent *A marginale* prevalence of 38.53% (95% CI, 33.26% to 43.81%) across all 4 farms (Table 1). We also estimated within-herd prevalence for each farm, which was 56.86% (95% CI, 43.27% to 70.46%) for the Coshocton farm, 49.41% (95% CI, 38.78% to 60.04%) for the Clinton farm, 33.87% (95% CI, 25.54% to 42.20%) for the Jackson farm, and 19.40% (95% CI, 9.93% to 28.87%) for the Lorain farm (Figure 1).

Our combined diagnostic approach identified 44 seropositive individuals that tested PCR negative for *A marginale*. Testing of these 44 individuals for *A phagocytophilum* revealed 2 (4.55%) positive detections.

Statistical analyses revealed support for both risk factors examined. Farm identity influenced infection status ($P < .001$); the odds an animal was positive for *A marginale* in Coshocton and Clinton was 5.48 (95% CI, 1.88 to 15.99; $P < .001$) and 4.06 (95% CI, 1.54 to 10.66; $P = .001$) times as high compared to the Lorain farm. Also, compared to Jackson, the odds an individual was positive in the Coshocton farm was 2.57 times as high (95% CI, 1.08 to 6.15; $P = .03$). While accounting for farm identity, we found that age was a significant ($P < .001$) predictor of infection status. Specifically, for every 1 year, the odds of becoming infected increased by a factor of 1.41 (95% CI, 1.28 to 1.58). Estimates of molecular prevalence for each farm were predicted with the multivariable model (Table 1). In summary, farm identity and animal age were significant risk factors for infection with *A marginale*.

Discussion

In this cross-sectional study, we characterized the prevalence of *A marginale*, the causative agent of bo-

vine anaplasmosis, in Ohio. Using serologic and molecular methods, we estimated a pooled prevalence of approximately 39%, ranging from approximately 19% to 57%, with older cattle more likely to be infected. This study was the first to document the prevalence of bovine anaplasmosis in Ohio and one of the few studies to do so by using 2 diagnostic methods. We also demonstrated serologic cross-reactivity between *A marginale* and *A phagocytophilum* by detecting 2 seropositive cattle that were PCR negative for *A marginale* but PCR positive for *A phagocytophilum*.

Evidence on the seroprevalence of bovine anaplasmosis in the US exists from various states, with estimates spanning a wide range. For example, true statewide seroprevalence in beef cattle ranged from approximately 29% in Mississippi,¹⁰ approximately 20% in Florida,⁹ approximately 15% in Iowa,⁷ approximately 12% in Texas,¹³ approximately 9% in Kentucky,¹² to approximately 3% in Georgia.¹¹ However, because serologic testing may lead to cross-reactions with other *Anaplasma* spp, it is unclear whether these and similar studies capture true estimates of *A marginale* prevalence. Evidence on molecular prevalence of anaplasmosis is more limited. For example, molecular prevalence in beef cattle from Texas varied 0% to 82% depending on the herd.¹⁴ Hence, although our statewide molecular prevalence of approximately 39% seemed higher than seroprevalence estimates, this was likely because our sampling was biased toward herds with a history of clinical anaplasmosis. However, our within-herd molecular prevalence did vary from approximately 19% to 57%, indicating a narrower range in Ohio than herds in Texas. Nonetheless, to better describe statewide and within-herd prevalence of bovine anaplasmosis in Ohio, we still require wider and randomized epidemiological studies.

Farm identity was a significant predictor of infection with *A marginale*, but it is unclear why this was the case (ie, environmental, ecological, or herd management factors). Although sterile needles and insecticides were commonly used at all the farms we sampled (thus limiting mechanical transmission), perhaps other equipment such as ear tag devices, nose tongs, and tattooing equipment may not have been regularly sterilized, and insecticides used (eg, moxidectin, diazinon, and coumaphos) may not protect against the biting insects responsible (eg, *Tabanus* and *Stomoxys* dipterans).³ Importantly, because tick vectors (primarily *Dermacentor* spp) have been reported in all of the counties we sampled,²⁶ they may play a critical transmission role that warrants more research. Interestingly, the Jackson farm was the only one with a closed herd, suggesting the possibility of intrafarm transmission through chronic carriers. Taken together, we would need larger targeted studies to understand why farms vary in their prevalence of *A marginale* and delineate what transmission route is most prevalent across farms in Ohio.

Although we found that older animals were more likely to be infected, this may not be unexpected because *A marginale* induces a chronic infection that can be detected with molecular techniques.¹ This finding supports previous studies from Texas,¹³ Kentucky,¹²

and California.⁸ Hence, our study lends further support to animal age as a risk factor for bovine anaplasmosis, although we do caution that some age data (the Clinton farm) may have been subject to recall bias.

The combination of serologic and molecular testing identified 44 seropositive individuals that tested PCR negative for *A marginale*. Testing of these individuals revealed that 2 were infected with *A phagocytophilum*. Infection of cattle with the North American strains of this agent is uncommon and leads to mild disease with seroconversion.²⁷ Therefore, although seropositive animals that were PCR negative for both *Anaplasma* spp could have had *A marginale* loads below assay detection, it is also likely they had cleared acute infection with *A phagocytophilum* but remained seropositive. Given these findings, we caution the use of serology alone for diagnosing *A marginale* infection wherever the 2 *Anaplasma* species co-occur. As the range of the tick vector of *A phagocytophilum* continues to expand, combining serologic with molecular techniques may become necessary to correctly diagnose bovine anaplasmosis in North America.

Out of the 29 cattle from the Coshocton farm that had been treated about a month prior to sampling with oxytetracycline (1 to 3 doses), 25 of these were PCR positive for *A marginale*. Although a small sample size, these findings corroborate previous studies,⁴⁻⁶ which suggest that antimicrobial therapy may not eliminate carriers. Therefore, carrier status after antimicrobial therapy should be scrutinized and not assumed.

We acknowledge that our study had limitations stemming from the objective of the broader study, which was to characterize species and genotypes of *Anaplasma* in Ohio. Given that we did not employ probability-based sampling, we cannot conclude that our findings represent other cattle herds in Ohio. We also cannot delineate what factors may have influenced within-herd prevalence at the farms we sampled. However, our study was the first to characterize the prevalence of bovine anaplasmosis in Ohio through a combination of diagnostic tests, thereby providing foundational knowledge for guiding wider and randomized epidemiological studies.

In conclusion, we estimated a molecular prevalence of approximately 39% across 4 beef cattle herds in Ohio, a state with no reported estimates in the literature. Combining serologic and molecular diagnostic methods allowed us to generate a more robust estimate of *A marginale* prevalence, an approach that can be used to guide similar prevalence studies elsewhere. Going forward, we need probability-based studies to further characterize the epidemiology of bovine anaplasmosis in Ohio and help inform prevention and control efforts in the state and the surrounding region.

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