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Surveillance of Anaplasma marginale in Arkansas Beef Cattle Herds

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Abstract

Anaplasmosis is an economically devastating disease in cattle that is caused by the rickettsial pathogen *Anaplasma marginale*. It is estimated that this parasitic bacterium causes over $300 million in expenses for the U.S. cattle industry annually. In Arkansas, the beef cattle industry is the fifth largest agricultural commodity in the state, thus necessitating a better understanding of this disease along with its prevalence. In this study, both polymerase chain reaction (PCR) and competitive enzyme-linked immunosorbent assay (ELISA) tests were used to determine the prevalence of *A. marginale* infection in Arkansas beef cattle on pasture in the six commonly known geographical regions within the state. Rates of regional seroprevalence and/or PCR prevalence ranged from 36.7% to 93.8% on samples obtained from 578 live beef cows that were two years of age or older. Overall, the highest percent prevalence was found along Crowley's Ridge in the northeastern corner of the state. Regional percentages were applied to a state map identifying the geographical regions for distribution to county extension agents within the University of Arkansas System Division of Agriculture for educational purposes. Data from this study will also be used to determine which strains of *A. marginale* are present in Arkansas with the possibility of developing novel therapeutic interventions in the future.

Key Words: Anaplasmosis, Beef cattle, PCR, ELISA, Anaplasma marginale, Prevalence

Introduction and Literature Review

Bovine anaplasmosis is a hemoparasitic disease that is found in many cattle throughout the world and is considered to be the one of the most significant diseases of cattle in the United States (Whitlock et al.; Torioni De Echaide et al. 1998; Kocan et al., 2010; Molly et al., 1999). First observed in South African cattle by sir Arnold Theiler in 1910 (Theiler. 1910a, 1910b and 1911), and then later officially recognized in a group of U.S. cattle from southeastern Kansas in 1926 (Darlington. 1926), bovine anaplasmosis is caused by the rickettsial pathogen, *Anaplasma marginale* (Kocan et al. 2010). This organism reproduces within the red blood cells of infected cattle, causing cell lysis, which results in generalized anemia and subsequent oxygen deprivation throughout the animal's body. The inability to transport oxygen throughout the body can then lead to a variety of health issues. Symptoms of infection may include depression, anorexia, fever, decreased feed efficiency or weight loss, abortion and death (Torioni De Echaide et al. 1998; Kocan et al., 2010; Hairgrove et al. 2010; OIE Terrestrial manual). Once infected most animals remain carriers for the remainder of their life as the animal is unlikely to completely clear the infection on its own. Because this pathogen lives within the red blood cells of the animal, clinical infection only becomes apparent once the immune system recognizes a significant pathogenic load within the blood and begins filtering the infected erythrocytes resulting in subsequent anemia. Cattle over the age of two years are the most susceptible to severe disease and death (Felsheim et al. 2010) while most young cattle under one year of age are not clinically affected due to their body's natural ability to rapidly replace infected erythrocytes (Hanzlicek, 2018a).

A. marginale is also known to colonize in the midgut and salivary glands of ticks (L.L. Hungerford. 1999), specifically the *Dermacentor andersoni* and *Dermacentor variabilis* ticks which are common in the U.S. (Kocan et al., 2010). Cattle are most often infected when the ticks move from animal to animal, transmitting the pathogen via saliva when feeding (Torioni De Echaide et al. 1998; Kocan et al., 2010 OIE Terrestrial manual). Infected cattle may then serve as biological reservoirs, harboring the pathogen, and thus allowing a substantial mechanical vector, i.e. biting flies, to pass the disease throughout the cattle population when fly populations surge (Hairgrove et al. 2010). Because it only infects ruminant species, wild ruminants, such as white-tailed deer, black-tailed deer, mule deer, Rocky Mountain elk, and American bison have also been shown to serve as reservoir hosts (reviewed by Kocan et al. 2010). These wild ruminants present year-round opportunities for passing of infection between animals and herds (L.L. Hungerford. 1999; Kocan et al., 2010). Additionally, previous work indicates that blood-contaminated multi-use injection needles, commonly used by producers, can also serve as a source of iatrogenic mechanical transmission between animals within the same herd (Hanzlicek, 2018b).

Diagnosis of *A. marginale* infection can be achieved through a variety of commonly recognized diagnostic tests including: Geimensa-stained blood smear; complement fixation, indirect immunofluorescence, card agglutination, serologic detection of antibodies via enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) (Torioni De Echaide et al. 1998; Kocan et al., 2010; OIE Terrestrial manual). This project chose to utilize both a competitive enzyme-linked immunosorbent assay (cELISA) and polymerase chain reaction (PCR) for the detection of *A. marginale* in private producer-owned and university-owned, Arkansas beef cattle. These tests were chosen based upon their abilities to provide

reliable detection of early infections, active parasitic infections and past exposure (Whitlock et al.; Hanzlicek, 2018b; De la Fuente, 2001).

Economically, anaplasmosis can have a variety of detrimental effects on the average cattleman's operation. The biological effects seen in cattle range from maintenance of a persistent carrier state, which may result in poor reproductive performance, to animals that experience sudden shock-induced death. In 2012, *A. marginale* was estimated to cost the U.S. cattle industry over $300 million (Whitlock et al., 2014). Recent changes to the Veterinary Feed Directive (VFD) that took effect in January of 2017 have restricted producer access to in-feed antibiotic treatments. A current VFD and a valid veterinary client patient relationship (VCPR) are now required to purchase and use in-feed antibiotics to control active bovine anaplasmosis (Federal Register). While this may cause difficulties for the cattle industry moving forward, full clearing of the infection by use of antibiotics is not necessarily the most efficient form of disease control for anaplsmosis. With the declining number of large animal veterinarians in many rural areas, along with the added cost associated with veterinary diagnostics, many cattle owners may not be able to afford or gain access to the necessary treatment options and an alternative method of disease prevention must be utilized.

At this time, vaccination is a form of disease control that requires further exploration due to the many strains and sub-strains of *A. marginale* that have been identified. The few vaccine options that have been produced over the years appear to mitigate acute symptoms of disease, but have not been able to prevent actual infection with *A. marginale* (Palmer et al. 1999). In 1998, the only two USDA-licensed and approved vaccines in the United States, one from Fort Dodge (Anaplaz) and the other from Mallinkrodt (Plazvax) (Maas, 1998), were removed from the

market. Since then, only an experimental killed vaccine (University Products, Baton Rouge, LA) has been made available in some states.

Because beef cattle are the fifth largest agricultural commodity in the state of Arkansas, with over 890,000 individual animals and growing (Arkansas Farm Bureau), these assets need to be protected for the sake of animal well-being and economic prosperity for the producers by all legal and ethical means available.

The objective of this study, is to provide a current state-wide perspective on the prevalence of *A. marginale* infection within Arkansas beef cattle. Theoretically, different geographical regions of the state may vary in the prevalence of *A. marginale* within cattle populations as a direct result of differences in vector populations, eco-climatic variables, management practices and socio-economic variables. Here we focus on eco-climatic differences and evaluated the prevalence of *A. marginale* in beef cattle from six different eco-climatic geographical regions: the Ozark Mountain region, the Arkansas River Valley region, the Ouachita Mountain region, the Coastal Plain region, the Crowley's Ridge region, and the Mississippi Alluvial Plain region (Frank, Dowling, 2019). Information gained from this study will ultimately be distributed to agricultural agents and producers across the state to further develop awareness and educational opportunities for discussion on best management practices, prevention and treatment. Furthermore, the future delineation of specific regional *A. marginale* strains can be used for the development of strain-specific vaccines that can protect Arkansas cattle from this devastating disease.

Materials and Methods

Sampling Strategy

Using United States Geological Survey data, samples were accepted based on their geographic location within one of the six geographical regions of Arkansas. With 10 farms from each of the regions submitting 10 different samples to be tested, 600 samples were intended to be accepted from across the state. This was the maximum number of samples that could be tested based on the provided budget.

Blood Sample Collection

All samples were collected in compliance with University of Arkansas IACUC protocol #19112. A total of 1,730 blood samples were collected from 578 Arkansas cattle, from 58 different farms, across 6 different geographical regions within the state. Of the samples collected, approximately 1,156 samples were serum and 547 were whole blood. The study was advertised via e-mail announcements and phone calls to University of Arkansas Division of Agriculture county agents, local publications, area Cattlemen's Association meetings and word of mouth to area beef cattle producers. A formal letter announcing/describing the study, bovine anaplasmosis factsheet, study flyer, producer consent form, blood sampling instruction sheet and producer survey were all sent electronically to 75 Division of Agriculture Cooperative Extension county agents in the fall of 2019. Agents and producers were specifically instructed to only sample beef cattle, two years of age and older, with no known history of anaplasmosis vaccination. A maximum of 10 head from each farm were to be randomly selected and sampled. Once a willing producer was identified, a blood collection kit(s) was distributed to the corresponding county agent, along with study-specific, county-farm-animal identification numbers for the animals being sampled. Each blood collection kit contained the following: 22, 7mL blood collection tubes without additive; 11, 7mL blood collection tubes with 7.2 mg

K_2 EDTA (Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ); 12, 18-guage blood collection needles (Vacuette needle; Greiner Bio-One North America Inc., Monroe, NC); 1, 5- blood collection needle holders (Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ) ; 1 pair of large, disposable nitrile exam gloves (Neogen, Lexington, KY); 1 pair of disposable boot covers (Continental Plastic, Delavan, WI); 2 copies of the producer consent form; and 1 copy of the blood collection/sampling instruction sheet. Agents and/or producers were instructed to collect two 7mL blood samples into blood collection tubes without additive and one 7mL blood sample into a blood collection tube containing K_2 EDTA from each animal identified to participate in the study via jugular venipuncture or tail venipuncture. For larger herds, it was requested that every third animal through the chute be selected for sampling, so as to get a thorough cross-section of the herd. Participants were instructed to invert the lavender-top tube at least 5 times to mix the blood with the EDTA for blood clotting prevention. Once samples were collected, they were kept on ice packs and/or refrigerated until transport, along with the producer consent form, to the University of Arkansas Fayetteville campus. Samples were generally received and processed within 1-7 days of collection.

Samples were received from November 1st, 2019 through February 21st, 2020. Upon arrival to the University of Arkansas Fayetteville campus, the whole blood and clotted blood tubes were sorted and labeled The lavender-top (whole blood) samples were logged and subsequently shipped in insulated shipping containers to the Reif Lab at Kansas State University College of Veterinary Medicine via overnight FedEx. The red-top (serum) samples were centrifuged at room temperature at 3,000 rpm for 20 minutes. The serum was then carefully pipetted into two 1.5ml Eppendorf tubes that were individually labeled with the previously assigned county-farm-animal identification number and stored in a freezer at -28.8ºC.

Pathogen detection

Genomic DNA (gDNA) was extracted from 100 µl samples of whole blood using the Quick-gDNA™ Miniprep Kit (Zymo Research, Irvine, CA) according to manufacturer recommendations. Final gDNA samples were eluted in 35 µl of DNA Elution Buffer and stored at -20°C. A quantitative real-time PCR (qPCR) assay targeting a portion of the single-copy, Msp5 gene was used to detect and quantify *A. marginale* in cattle blood samples as previously described (11). Briefly, qPCR reaction mixtures consisted of the following in a 20-µl total volume per reaction: 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.2-µM Am msp5-F primer (5' – ATA CCT GCC TTT CCC ATT GAT GAG GTA CAT – 3'), 0.2-µM Am msp5-R (5' – AGG CGA AGA AGC AGA CAT AAA GAG CGT – 3'), and 2-µl gDNA. Amplification was performed using a CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: 98°C for two minutes, 40 cycles of 98°C for 5 seconds, 60°C for 5 seconds, and 74°C for 15 seconds; and a final melting curve step (65°C to 95°C in 0.5°C increment steps at 5-sec per step). CFX Maestro Software (Bio-Rad Laboratories, Hercules, CA) was used to display results.

Antibody Detection

The ELISA tests were completed over two days at the University of Arkansas Division of Agriculture Veterinary Diagnostic Laboratory using the Veterinary Medical Research & Development (VMRD, Pullman, WA) *Anaplasma* Antibody Test Kit, cELISA v2 according to manufacturer recommendations (VMRD, Pullman, WA).

Statistical Analysis

Binomial data were analyzed using the FREQ procedure and quantitative means were analyzed using the GLIMMIX procedure of SAS version 9.4 (SAS Inst. Inc., Cary, NC). The chi-square statistic was utilized to determine independence of homogeneity between positive and negative cows either statewide or within each geographic region. Means of prevalence rates were separated with a t-test using the PDIFF option in SAS, and a *P*-value $\leq$ 0.05 was considered significant.

Regional Map Construction

The regional farm participation map was created using a standard Arkansas map template, then adding markers using the Microsoft PowerPoint program. The regional prevalence map was created by using a standard Arkansas map template with the designated geographical regions. Colorization and labeling were added with the Adobe Illustrator® program.

Results

Blood samples were collected from cattle representing 58 farms spread out across the state of Arkansas. Blood samples were drawn from approximately 10 head of cattle at each farm, for a total of 578 animals tested. Of the total samples submitted, 578 cELISA tests and 574 qPCR tests were completed. Among the cELISA tests completed, 300 (51.9%) tested seropositive (percent inhibition $\geq$ 30%) for *A. marginale*. Among the qPCR tests completed, 267 (46.5%) tested positive for *A. marginale*. If at least one of the tests was not completed for an animal, due to blood clotting or lack of submission, then data for those samples were removed for subsequent comparisons, resulting in a total of 573 animals with samples tested by both cELISA and qPCR. Out of the total 573 individuals in which comparative data was available,

335 (58.5%) individual animals tested positive for *A. marginale* on at least one of the two tests, while 238 (41.5%) were negative on both tests. Of the 335 animals positive for anaplasmosis by cELISA and/or qPCR, a total of 229 individuals (68.4%) tested positive on both tests, while 68 individuals (20.3%) tested positive on cELISA only, and 38 individuals (11.3%) tested positive on qPCR only.

The geographical distribution of the 573 individuals tested, in which both cELISA and qPCR were able to be run, is as follows: 115 head (20.1%) from the Ozark Mountain (OZM) Region; 110 head (19.2%) from the Coastal Plain (CPL) Region; 100 head (17.5%) from the Mississippi Alluvial Plain (MAP) Region; 89 head (15.5%) from the Arkansas River Valley (ARV) Region; 80 head (14.0%) from the Crowley's Ridge (CWR) Region; and 79 head (13.8%) from the Ouachita Mountain (OUA) Region.

Within each region, the following numbers were observed regarding the number of individuals that tested positive for *A. marginale* on at least one of the two (cELISA and/or qPCR) tests: 43 head (37.4%) in the OZM Region; 56 head (50.9%) in the CLP Region; 85 head (85%) in the MAP Region; 47 head (52.8%) in the ARV Region; 75 head (93.8%) in the CWR Region; and 29 head (36.7%) in the OUA Region.

Conclusions and Discussion

The decision to separate the state into the six geographical regions was based on variables thought to influence *A. marginale* vector populations. Both the Ozark Mountain Region and Ouachita Mountain Region, though increased in elevation, are also densely wooded, and thus were initially expected to have a higher incidence of infected cattle due to the increased probability of individual exposure to ticks. However, these two regions demonstrated the lowest

concentration of positive cattle with 37.4% and 36.7% respectively, making up a combined 21.5% of the total positive cattle detected statewide.

Alternatively, the highest incidence of *A. marginale* infection relative to other regions was found in cattle within the Crowley's Ridge and Mississippi Alluvial Plain Regions of the eastern Arkansas, with 93.8% and 85.0% positive rates respectively. Combined, these regions accounted for 47.8% of the total positive cattle tested statewide. Small in size and surrounded on the east and west by the Mississippi Alluvial Plain, the Crowley's Ridge Region is specifically known for its high number of beef cattle operations because of its lack of usable row crop lands. It is hypothesized that the large populations of biting flies and mosquitoes associated with the flat, waterlogged regions of eastern Arkansas might be significant contributors to the spread of *A. marginale* among beef cattle in the Eastern portions of the state. It is also postulated that the superior mobility of the flying/airborne vectors may serve to facilitate the movement of infection between animals and herds more efficiently than the biological vector, the tick. Further research into the rate of transmission by the different vectors would be valuable.

The two geographic regions situated in the middle of our statistical analysis were the Arkansas River Valley Region and the Coastal Plain Region, with 52.8% and 50.9% positive rates respectively. Combined, these two regions accounted for 30.8% of the total positive cattle tested statewide. While both the Arkansas River Valley and Coastal Plain sit at lower elevations compared to the mountain regions, it should also be noted that the Coastal Plain is generally densely wooded and typically warmer than other parts of the state, thus providing opportunity for various potential vectors to inhabit the region.

Overall, there were statistically significant differences ($P \leq 0.05$) among all of the geographic regions, except between the previously mentioned Ozark Mountain and Ouachita

Mountain Regions; the Mississippi Alluvial Plain and Crowley's Ridge Regions; the Arkansas River Valley and Coastal Plain Regions; and the Ouachita Mountain and Coastal Plain Regions.

The majority of individuals tested (467 of 573; 81.5%) had concordant qPCR and cELISA test results. However, tests on 18.5% (106 of 573) of individuals tested resulted in discordant results, meaning that one test was positive while the other test was negative.

In regards to test results where individuals were qPCR positive and cELISA negative (68 of 573; 11.9%), the results indicate that these animals are actively infected with *A. marginale*, but there are not sufficient blood antibody levels to *A. marginale* to indicate significant immune response. Reasoning for this particular outcome might include that these individuals have been recently infected with *A. marginale* and the initial antibody response, or seroconversion, had not yet occurred. Infected individuals may be infected for up to a month before seroconversion, detectable via cELISA, occurs.

In regard to test results where individuals were qPCR negative and cELISA positive (38 of 573; 6.6%), the results suggest that these individuals are not actively infected with *A. marginale*, but do have an antibody response indicative of previous infection or exposure to the infectious organism. It is hypothesized that these individuals may have cleared their infection spontaneously or with the help of antibiotic treatment. These results might also indicate that the animal had previously received vaccination for *A. marginale*. For the purposes of this study, based upon the requirements for participation in the study, and because use of the vaccine is not known to be widespread in the state, these individuals were counted as generally "positive". This means that infection was most likely present for a period of time sufficient enough for the individual to mount an immune response.

It should be noted that PCR testing is the standard confirmation test used to detect active infection of *A. marginale* in infected cattle, while ELISA testing is best used for general herd surveillance. Comparatively, ELISA testing is significantly lower in cost for producers to monitor their herds. In Arkansas, 96% of the beef cattle industry in the state is categorized as cow-calf operations containing 50 head or less of cows with one or more bulls (Arkansas Farm Bureau). As a result, many producers may not generate enough revenue through their operation to justify the cost of herd PCR testing.

This research uncovered particularly interesting results that could have a variety of effects on the beef cattle industry. With over 58% of the beef cattle tested in this study having positive test results, there are significant economic impacts that should be considered. Given the new implementations to the VFD, the short-term and long-term financial implications of this disease need to be assessed and recognized as a substantial hurdle for producers with both small and large herds.

While this research was able to address the prevalence of *A. marginal* within the six major geographical regions of Arkansas, the full significance of these findings is still yet to be determined. Areas for future investigation include a closer look at the various environmental factors that could be playing a role in pathogen prevalence within herds of certain regions. Additional opportunities also include evaluation of vector species population densities associated with the geographical areas, ability of those vector species to effectively transmit the disease, assessment of long-term reproductive performance by infected/carrier animals, identification of regionally specific *A. marginale* strains, as well as regional assessment of producer management practices. While disease in general is often a difficult and ambiguous issue for many producers to address, such specific information as it pertains to *A. marginale* in the state of Arkansas would

hopefully help to further establish best management practices for the most efficient control of bovine anaplasmosis through the effective preventative, diagnostic and treatment measures.

Acknowledgements

Blood kit acquisition and blood sampling were coordinated by Dr. Heidi Ward, the livestock Extension veterinarian for the University of Arkansas System Division of Agriculture. Dr. Ward also distributed information about the study to the agriculture agents and producers and promoted the study with the Arkansas Farm Bureau and Arkansas Cattlemen's Association.

Coordination of blood sampling within in many of the Coastal Plain counties was led by Dr. Charles Looney. Blood sampling from herds within the University of Arkansas System were conducted by Dr. Jeremy Powell, Dr. Rocky Lindsey and Don Hubbell. Dr. Powell also helped with experimental design and manuscript editing.

All qPCR tests were conducted and analyzed at the Kansas State University College of Veterinary Medicine, with the assistance of Tippawan Anantatat and Macy Flowers, under the supervision of Dr. Kathryn Reif.

All cELISA tests were conducted and verified at the University of Arkansas Division of Agriculture Veterinary Diagnostic Laboratory, with the assistance of Alicia White, under the supervision of Dr. Randall Moore.

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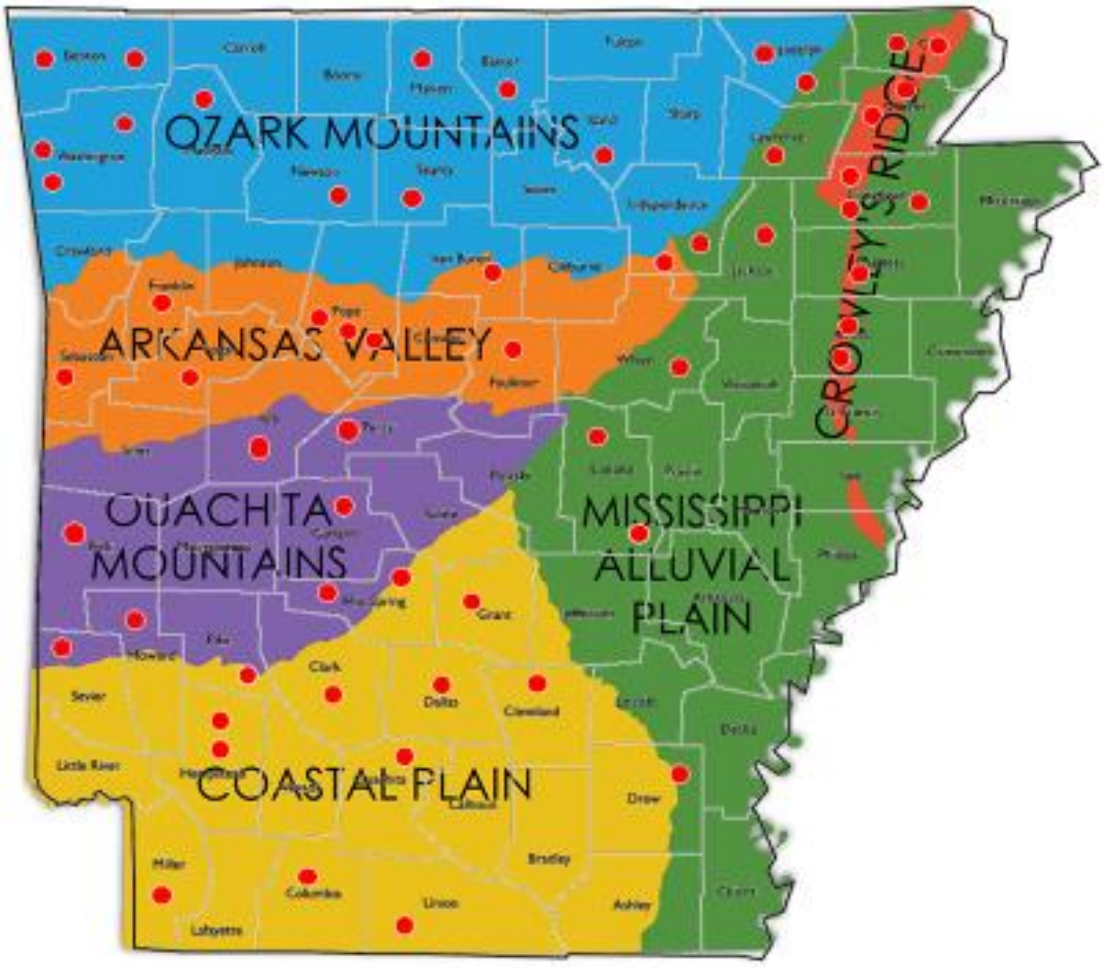
Figures

Figure 1

Geographic Regions & Locations of Participating Farms

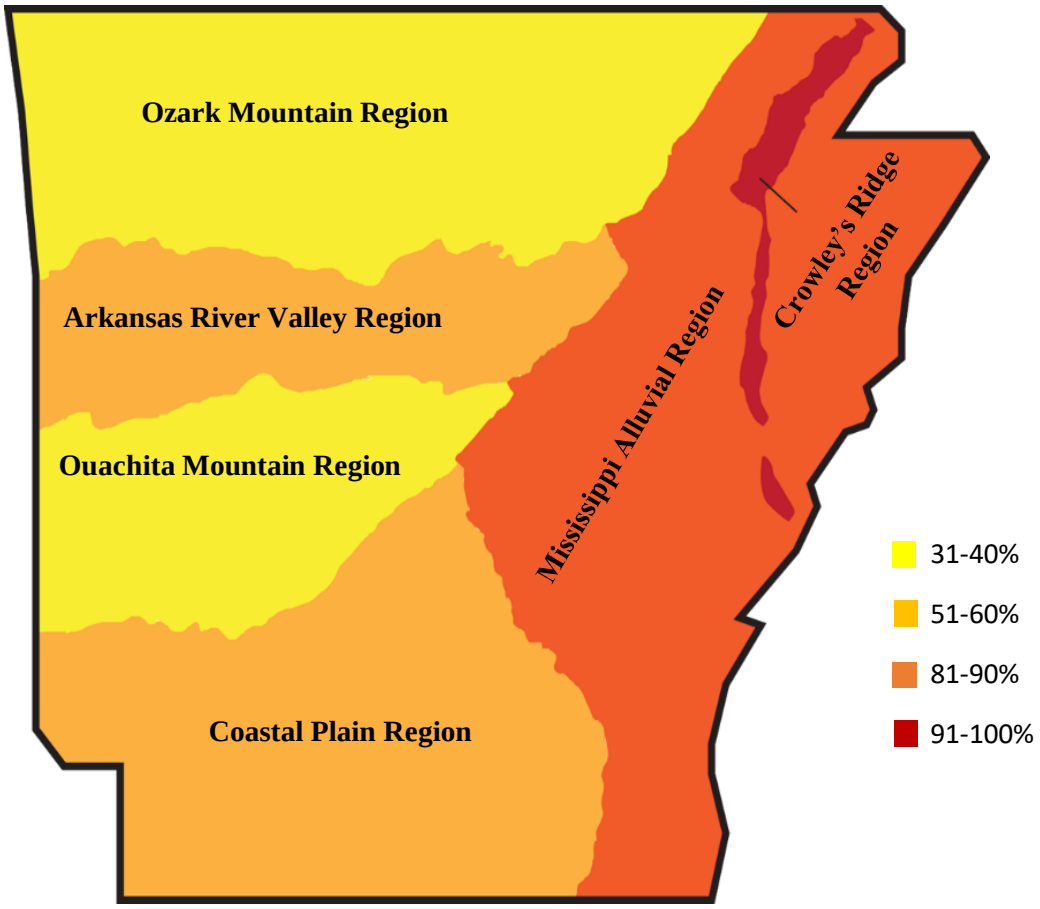


Key: ● = Participating Farm Location

Note: Total Number of Participating Farms per Region: Ozark Mountain Region (OZM) = 12; Arkansas River Valley Region (ARV) = 9; Ouachita Mountain Region (OUA) = 8; Coastal Plain Region (CLP) = 11; Mississippi Alluvial Plain Region (MAP) = 10; Crowley's Ridge Region (CWR) = 8.

Figure 2

Regional Prevalence of Anaplasmosis in Arkansas Beef Cattle



Note: Concentration of positive *Anaplasma marginale* tests in Arkansas beef cattle by geographic region.

Tables

Table 1

Comparative ELISA and PCR Test Results

Result Classification	(#) Results	(%) Results	Cumulative (#+) Results	Cumulative (%+) Results
(-ELISA,-PCR)	238	41.54%	-	-%
(+ELISA,+PCR)	229	39.97%	229	39.97%
(-ELISA,+PCR)	38	6.63%	267	46.60%
(+ELISA,-PCR)	68	11.87%	335	58.47%
Total	**573**	**100%**	**335**	**58.47%**

Note: Individual animal test results based up paired cELISA and qPCR results.