PEER REVIEWED

Review of epizootic hemorrhagic disease in cattle and a study defining seroprevalence of epizootic hemorrhagic disease virus serotype 2 in Texas cattle

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Abstract

Epizootic hemorrhagic disease virus is an orbivirus transmitted by *Culicoides* spp. In North America, it is recognized as a major cause of disease affecting white-tailed deer, but it can impact other wildlife species as well as domestic cattle. Although minimal clinical disease had been noted in Texas, periodic outbreaks in other states caused Texas cattle producers to question the risks to their cattle. The objective of this study was to estimate the proportion of Texas cattle exposed to epizootic hemorrhagic disease virus serotype 2 by observing seroprevalence in auction markets. Serum samples collected from cattle ≥18 mo of age sold through 11 Texas auction markets were collected during June of 2014. Antibody levels were measured using the virus neutralization test as the diagnostic protocol. Market sampling indicated 97.08% of adult cattle had been exposed to epizootic hemorrhagic disease virus serotype 2. There were no significant differences in mean titers between individual markets. However, when markets were grouped there was a significant mean titer difference between groups, increasing in the southern and western regions. The lack of clinical disease is likely related to enzootic stability as a result of high viral infections in cattle and white-tailed deer and the abundance of *Culicoides* vectors.

Key words: seroprevalence, cattle, EHD, epizootic hemorrhagic disease virus, *Culicoides*

Résumé

Le virus de la maladie hémorragique épizootique appartient au genre Orbivirus et est transmis par *Culicoides spp.* En Amérique du Nord, ce virus constitue une cause majeure de maladie chez le cerf de Virginie mais il peut aussi affecter d'autres animaux de la faune de même que des bovins domestiques. Bien que peu de cas cliniques de maladie ont été observés au Texas, des foyers de maladie dans d'autres états ont amené les producteurs du Texas à se questionner sur les risques pour leur bétail. L'objectif de cette étude était d'estimer la proportion de bovins du Texas exposés au virus de la maladie hémorragique épizootique de type 2 en mesurant la séroprévalence dans des encans. Des échantillons de sérum ont été recueillis chez des bovins âgés de 18 mois ou plus vendus dans 11 encans en Juin 2014. Comme protocole diagnostic dans cette étude, on a utilisé l'essai de neutralisation virale pour mesurer les niveaux d'anticorps. L'échantillonnage des encans a montré que 97.08% des bovins adultes avaient été exposés préalablement au virus de la maladie hémorragique épizootique de type 2. Il n'y avait pas de différence significative au niveau des titres moyens entre les différents encans. Toutefois, lorsque les encans ont été combinés, les titres moyens dans les régions du sud et de l'ouest étaient significativement plus élevés. L'absence de maladie clinique est probablement reliée à l'enzootie stable découlant du niveau très élevé d'infection chez les bovins et les cerfs de Virginie et à l'abondance des vecteurs *Culicoides*.

Introduction

Epizootic hemorrhagic disease (EHD) is a non-contagious infectious viral disease of wild and domestic ruminants transmitted by biting midges of the genus *Culicoides*.[19,23,37] The causative agent, epizootic hemorrhagic disease virus (EHDV), is a double-stranded RNA virus belonging to the

Reoviridae family, genus *Orbivirus*.[5,37] Globally, there are 7 recognized serotypes of EHDV, with 3 serotypes found in North America.[4,35]

Since its isolation in North America in 1955, EHDV is considered an important viral agent affecting white-tailed deer populations in the United States, with outbreaks of sudden death occurring in summer and early fall, coinciding with vector seasonality.[b,30,38,41] The most commonly affected wildlife species in North America are white-tailed deer, but mule deer (*Odocoileus heminous*), elk (*Cervus elaphus*), big horn sheep (*Ovis canadensis*), and pronghorn (*Antilocapra americana*) have sporadically developed fatal disease.[10,23,30,32] Favero et al reported the only confirmed clinical case of EHD in wild ruminants outside of North America, occurring in a captive pygmy brocket deer (*Mazama nana*) in South America;[9] however, EHDV antibodies have been detected in numerous wildlife species.[34]

Epizootic hemorrhagic disease virus and bluetongue virus are antigenically different orbiviruses, but share common vectors and hosts. They also have comparable spatial and temporal distributions and produce disease in white-tailed deer that is clinically and pathologically similar, and can only be diagnosed by pathogen differentiation.[b,d,15,25,36,37]

Culicoides are responsible for transmitting bluetongue and EHD. Epizootic hemorrhagic disease virus is most often found between 45° latitude north and 35° latitude south, with viral enzootic stability being associated with more tropical regions.[d,23,28] *Culicoides variipennis sonorensis* is considered the primary vector in United States, but other *Culicoides* spp are associated with viral transmission in different geographical areas.[11,28,44] Increased environmental temperatures are more conducive to viral replication within the *Culicoides* spp vector.[27,36] Movement of the vector and/or movement of infected animals are associated with disease spread. Kedmi et al determined the 2006 EHDV outbreak in Israeli cattle was not associated with animal movement, but rather high-altitude wind movement of *Culicoides* spp vectors.[21]

Cattle readily seroconvert to EHDV, usually without observable clinical lesions, and while there is uncertainty, cattle probably amplify the virus, involving them in the epidemiology of EHDV in white-tailed deer.[5,15] While not generally considered a significant cattle disease, there have been sporadic national and international outbreaks of disease in cattle.[7,16,20,23,51] In 1955, hundreds of cases of a cattle vesicular disease were observed in southeastern Pennsylvania and Delaware at the same time a large-scale deer die-off was occurring in New Jersey.[17,29,42]

Epizootic hemorrhagic disease has been diagnosed in cattle, bison, and yak herds, with lesions suggestive of vesicular disease in Oregon, Tennessee, Colorado, Indiana, Illinois, South Dakota, and Nebraska.[6,12,18,44,48]

There are global reports of severe outbreaks of cattle EHD over the last 5 decades including Ibaraki virus, an EHDV-2 serotype found in Japan and Korea,[16] and recent cattle disease outbreaks of various EHDV serotypes on France's Reunion Island[7] and in Israel, Turkey, Morocco, Algeria, Jordan, and portions of North America.[20,23,51]

While EHDV-1 and EHDV-2 have been associated with epidemics in North American white-tailed deer for decades, EHDV-6 was first isolated in the United States from dead white-tailed deer in Indiana and Illinois during 2006,[2] and in subsequent years the virus has continued to be isolated over a larger geographic area in the United States.[b,3,10,35]

Pathogenesis of EHDV-2 infection in cattle is similar to other wild and domestic ruminants, with initial viral replication in the lymph nodes and lymphatic vessels that drain the area of vector inoculation.[23,25,37] The virus is then disseminated to secondary sites, replicating in the endothelial cells of tissues, such as lung and spleen, causing vascular injury and associated intravascular coagulation, resulting in hemorrhage, edema, and tissue necrosis with vesicular lesions noted on the gums, tongue, udder, and feet.[23,47] The virus affecting cattle is associated with the cell fractions of the blood, especially the erythrocytes, resulting in a prolonged viremia which provides a source of continued infected vectors.[1,15,23,37] Cattle serving as amplifying hosts are involved in the epidemiology of EHDV, and because sampling of individual wildlife is usually a single event, cattle can be employed as sentinel animals.[5,6]

A nationwide survey conducted from 1980 to 1989 measured morbidity and mortality in wild ungulates and found only 0.06% of reported wildlife (10 of 1,608) diagnosed with EHDV were of Texas origin. Nine of the reports were from the eastern part of the state, indicating disease variation based on geographical distribution with most of the cases being EHDV-2.[30,43] Texas A&M Veterinary Medical Diagnostic Laboratory data indicates limited EHDV-6 findings in deer (unpublished data). Stallknecht et al also noted the geographical distribution of EHDV when he serologically evaluated 685 white-tailed deer throughout Texas, with samples being collected over a 5-month period during the winter of 1991-1992.[43] State seroprevalence was 84%, but varied with ecological regions, increasing in a westerly direction with 100% seroprevalence in the northwest Edwards Plateau, which was considerably higher than the 57% observed in the Gulf Prairie region.[43] Increased exposure and seroprevalence were associated with a decrease in clinical disease as a result of a near perfect host-virus relationship believed to be related to enzootic stability.[14,24,43]

Nettles et al noted larger deer die-offs in temperate regions,[30] and deer mortality observed by Shultz in Wyoming[38] and Pasick et al in British Colombia[32] suggest that the lack of enzootic stability contributes to clinical disease in deer. A serological survey of auction market cattle in British Colombia and Alberta was conducted in the fall of 1987 following a disease outbreak in the Okanagan valley of British Colombia and indicated a seroprevalence of only 3%, indicating a lack of enzootic stability.[39] Enzootic stability, associated with more tropical regions, would explain high seroprevalence and lack of clinical disease associated with EHDV in Kenya, French Guiana, and northern Australia and the extensive disease

outbreaks in more temperate regions such as Israel, Morocco, Algeria, Jordan, and frequent epidemics of Ibaraki disease in Japan.[16,45,46,49,50] Gaydos et al observed differences in innate and acquired resistance among EHDV challenged white-tailed deer subspecies[14]; subspecies originating in more temperate climates (*Odocoileus virginianus borealis*) experienced higher mortality than subspecies from subtropical climates (*Odocoileus virginianus texanus*).[36] Extensive mortalities have been observed in farmed white-tailed deer moved from temperate to more tropical regions.[10] Deer challenged with EHDV-1 or EHDV-2 appear to be protected against clinical disease when later challenged with the same strain, and deer infected with EHDV-2 were protected against clinical disease when exposed to EHDV-1, but they still developed a viremia to the challenged EHDV serotype, indicating challenged deer serve as viral amplifying hosts.[b,13,33,42]

The objective of this study was to estimate the seroprevalence of EHDV-2 in Texas cattle using serum samples collected at 11 Texas auction markets for the purpose of brucellosis testing. This study only reports on the seroprevalence of EHDV-2 in cattle marketed through the 11 respective auction markets; however, the inference is that market seroprevalence is related to seroprevalence of cattle in the surrounding area. One limitation of the study was only cattle ≥18 months were evaluated, so seroprevalence in younger cattle was not measured. Seroprevalence in Texas cattle was hypothesized to be high, most likely due to enzootic stability because of the presence of the abundant disease vector *Culicoides* spp.

Materials and Methods

This study was approved by the Agriculture Animal Care and Use Committee-Texas A&M AgriLife Research (AUP 2014.022A).

Sample procurement

Serum samples were obtained from cattle sold at 11 auction markets located in cattle-dense regions of Texas (Figure 1). The auction markets were engaged in first-point brucellosis testing, requiring procurement of a blood sample from all breeding cattle ≥18 months of age. The executive director of the Texas Animal Health Commission (TAHC) granted permission for secondary use of these samples for investigating seroprevalence of EHDV. The markets were responsible for the collection of blood samples and shipment to the TAHC State-Federal Laboratory for confirmation of brucellosis test results. Serum samples were transported from the State-Federal Laboratory to the animal science department at Texas A&M University by common carrier for next-day delivery, then delivered to the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) where EHDV-2 virus neutralization assays were performed.

Previous TVMDL submissions anecdotally indicated EHDV-2 seroprevalence was greater than 50% in Texas cattle.

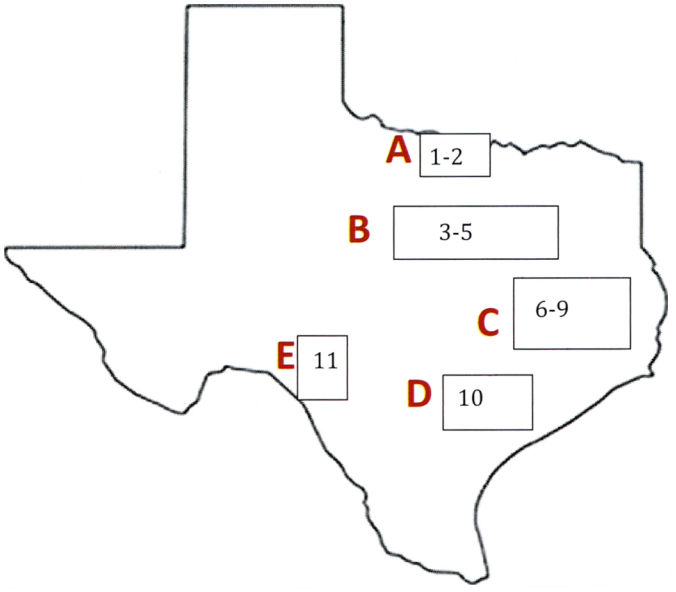

Figure 1. Locations of 11 Texas livestock markets (denoted by numbers 1-11) and their regional groupings (denoted by letters A-E).

Epi Info version 7.1.5[a] was used to determine the per-market sample size based on an estimated true proportion of 90%, a desired precision of 10%, and a 95% confidence level in a finite population of 2,677 market animals. Available samples from each market were numbered in sequence and an online random generator was used to select samples for testing from that market (Table 1).

Virus neutralization test

Fifty µl of 2-fold serial sera dilutions, from 1/10 to 1/1280, were added to each test well of flat-bottomed 96-well microtiter plates and each mixed with an equal volume

Table 1. Serum samples collected from 11 Texas livestock markets in June 2014. A total of 308 samples representing the 11 markets were analyzed.

Markets (North to South)	Total number of samples obtained per market	Sampled used in the analysis
1	396	32
2	360	32
3	353	32
4	120	27
5	303	31
6	606	33
7	82	25
8	88	25
9	17	12
10	199	30
11	153	29
Total	2,677	308

Note: Market sample size was determined using Epi Info version 7.1.5.

of known EHDV-2 serotypes (100 TCID50). The plates were incubated at 98.6°F (37°C) in 5% CO2. After 1 hour of incubation, approximately 10^4 Vero cells were added per well in a volume of 100 µl of minimal essential medium (MEM) containing antibiotics and, after incubation for 4 to 6 days, the test was read using an inverted microscope. Wells are scored for the degree of viral cytopathic effect (CPE) observed; a sample was considered positive when it showed 75% to preferably 100% CPE inhibition at the lowest dilution (1/10). The serum titer represents the reciprocal of the highest serum dilution capable of reducing more than 75% CPE in cell culture.[31] Sera were tested in duplicate.

Titers received from the TVMDL ranged from ≤20 to ≥1280, with no titer endpoints and values of ≥20 were considered positive. For analysis purposes, titers reported as <20 were assigned a value 10, the nearest lower dilution, and titers reported as ≥1280 were assigned a value of 2560, the next higher dilution. All data were entered into an Excel[c] spreadsheet and imported into Stata 14.1[e] for analysis.

Statistical analysis

The Texas Veterinary Medical Laboratory considers a titer ≥1/20 specific for EHDV-2. Data were analyzed to determine prevalence associated with each cutoff value. Titers were converted into binary data (negative=0, positive=1) and analyzed using a χ^2 test to determine differences in seroprevalence among markets at different titer cutoffs.

Mean titers were converted using log base 2 and assessed for normality across each market using the Shapiro-Wilk test.[40] Normality was assumed at $W \geq 0.9$. Converted titers were used to assess differences in mean titers across markets using a 1-way ANOVA. The Tukey-Kramer post hoc test was then used to assess the significance of pairwise comparisons between markets.[22]

Markets were grouped into 5 regions from north to south in order to determine regional differences (Figure 1). Mean titers across markets in different regions were analyzed using a 1-way ANOVA. The Tukey-Kramer post hoc test was used to assess the significance of pairwise comparisons between regions.

Results and Discussion

Seroprevalence studies have largely focused on the impact of EHDV on wild cervids and captive deer.[g,8,43] This study concentrates on seroprevalence of EHDV-2 in cattle moving through auction markets with the understanding the animals may have originated from other geographic areas; however, this is the most expedient method to sample different geographical regions of the state, and there is reasonable assumption that most of these cattle originated in proximity to the market. Results of this study demonstrated that EHDV-2 is prominent in Texas cattle.

Applying a cutoff of ≥20 resulted in a seroprevalence of 97.8% for all samples included in this study (Table 2).

Table 2. Seroprevalence of EHDV-2 in cattle >18 months of age marketed through 11 Texas livestock markets comparing different positive cutoff values derived from the virus neutralization test.

Titer cutoffs designated positive	Seroprevalence all markets
≥20=positive	97.08%
≥40=positive	94.81%
≥80=positive	94.10%
≥160=positive	87.66%
≥320=positive	69.16%
≥640=positive	44.16%
≥1280=positive	20.13%

Even when serial dilution titers ≥320 were evaluated as a positive titer cutoff, seroprevalence approached 70%. When titer cutoff values ≥80 were used, there were no significant differences in seroprevalence among markets (Table 3). In contrast, significant differences in titers were found between markets at titer cutoffs of ≥160.

Results indicate a difference among markets ($P<0.01$); however, no apparent patterns were discerned among markets as independent units. Thus, interpretations were based upon regional groupings. The lowest mean titers were in the northern part of the state and the highest mean titers were in the southern part of the state, with intermediate mean titers in central Texas as depicted in Table 4.

Even in endemic populations a seroprevalence of 97.2% measured at a titer cutoff of ≥20 is high. Cutoff titer values at each dilution were analyzed and approximately 70.0% of the population remained positive at a dilution titer of ≥320, 4 dilutions above the reported positive titer of ≥20. This is indicative of a high seroprevalence of EHDV in cattle moving through Texas auction markets.

Analyzing markets using generalized mixed modeling indicated there was a difference between markets, but determining which markets were different became problematic.

Clinical disease associated with EHDV-2 in Texas cattle is extremely rare, presumably due to enzootic stability as

Table 3. Chi-square analysis on each market for differences in titers across livestock markets. Cattle titers were similar across markets in the 3 lower dilutions, whereas in the higher dilutions markets were different.

Titer	Pearson chi-square	*P*-value†	Similar across markets
≥20=positive	13.54	0.20	Yes
≥40=positive	11.94	0.29	Yes
≥80=positive	17.24	0.07	Yes
≥160=positive	27.97	<0.01	No
≥320=positive	80.27	<0.01	No
≥640=positive	72.38	<0.01	No
≥1280=positive	56.76	<0.01	No

†Value of 0.05 was used for α in this assessment.

Table 4. Mean transformed titers across regions in Texas determined using a one-way ANOVA of mean titers with mean separation performed by pairwise *F*-protected *t*-test with Tukey-Kramer adjustment. Mean transformed titers in extreme north Texas markets differ from south east/west markets but mean transformed titers in central north/south markets are similar to all markets ($P<0.05$).

Regional clustering (see Figure 1)	Mean Titers
Extreme North Texas (Markets 1 and 2)	8.28[b]
Central North Texas (Markets 3-5)	8.32[a,b]
Central South Texas (Markets 6-9)	8.48[a,b]
South East Texas (Market 10)	9.34[a]
South West Texas (Market 11)	8.78[a]

a result of vector concentration. Likewise, clinical disease in cervids and cattle increases in the northern latitudes due to the presumed lack of enzootic stability associated with erratic populations of *Culicoides* spp.[h] In areas where seroprevalence is high, occurrence of clinical disease is rare because a large portion of the population possess circulating protective antibodies.

The trends in mean titer levels observed in this study are consistent with the concept of enzootic stability, with mean titers increasing towards southern latitudes very similar to a previous study on EHDV and BTV in deer.[43] Cattle also serve as amplifying hosts, and with 10.8 million cattle in Texas one could argue cattle density contributes to enzootic stability. However, Texas land mass is 261,797 square miles, while Kansas and Nebraska have combined cattle numbers similar to Texas, but their land mass is only 158,687 square miles. Kansas and Nebraska have greater cattle density, fluctuating vector populations, and sporadic outbreaks of clinical disease associated with EHD, indicating that the largest contribution to enzootic stability is most likely consistent exposure to vectors.

Conclusions

As with other vector-borne diseases, cattle producers should be aware that movement of naive cattle into endemic areas with abundant vectors could result in clinical disease. Nationwide movement of cattle has increased in the last decade as a result of drought. Moving cattle from temperate to tropical regions could conceivably be problematic, introducing naïve cattle into areas with high pathogen load and abundant vectors. A licensed vaccine is not currently available, but if required an inactivated product should provide sufficient immunity.[26] Although this study focused on auction-market cattle, information gained from this study provides the Texas cattle industry with an indication of EHDV-2 prevalence and its association with the stability of the *Culicoides* spp vector.

Endnotes

[a]Epi Info, version 7.1.5, Centers for Disease Control, Atlanta, GA

[b]Hecht AN. Temporal and spatial distribution of antibodies to a novel epizootic hemorrhagic disease virus (EHD-6) in white-tailed deer populations in the eastern US. Unpublished master's thesis, 2010. University of Georgia, Athens.

[c]Microsoft Excel 2010, Microsoft Corp, Redmond, WA

[d]Schoenthal CA. Monitoring and management of *Culicoides* spp (*Diptera Ceratapogonidae*) in white-tailed deer (*Odocoileus virginianus*) production facilities in Texas, USA. Unpublished doctoral dissertation, 2015. Texas A&M University, College Station.

[e]Stata 14.1, StataCorp, College Station, TX

[f]American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA

[g]Weaver JH. The role of parasites, diseases, mineral levels, and low fawn survival in a declining pronghorn population in the Trans-Pecos region of Texas. Unpublished master's thesis, 2013. Sul Ross State University, Alpine, TX.

[h]SDSU tracks epizootic hemorrhagic disease in South Dakota cattle. Animal Health Matters. 2012;5:1, 3.

Acknowledgement

The authors declare that there are no conflicts of interest.

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