RISK ANALYSIS OF BLUETONGUE VIRUS IN WILD UNGULATES IN WYOMING USING GIS

Final Report to the Wyoming Wildlife\Livestock Diseases Research Partnership.

ABSTRACT

Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) cause hemorrhagic disease, a noncontagious and vector-borne disease of wild and domestic ruminants including domestic sheep (*Ovis aries*), domestic cattle (*Bos taurus*), white-tailed deer (*Odocoileus virgineanus*), mule deer (*Odocolileus hemionus*), pronghorn (*Antilocapra americana*), elk (*Cervus canadenisis*), and bighorn sheep (*Ovis canadensis*). In Wyoming these viruses have been isolated from all these species. Both viruses are transmitted by species of *Culicoides* (Diptera: Ceratopogonidae) biting midges. While the viruses are well researched in the laboratory, less is understood about the geospatial factors that contribute to natural disease outbreaks in the western United States. The objectives of this study were to 1) determine presence or absence of antibodies against bluetongue virus in pronghorn, mule deer, and white-tailed deer as an indication of prior virus exposure; 2) test positive samples for specific antibodies against native bluetongue serotypes 11 and 17; and 3) correlate location specific seroprevalence, as the indication of prior infection, with predictor variables of species, elevation, slope, aspect, average summer precipitation, average maximum and minimum temperature, proximity to roads, proximity to water, and land cover classification to create a risk model for virus exposure. Wildlife blood samples were collected on Nobuto blood filter paper in the falls of 2011 and 2012 from hunter harvested pronghorn, mule deer and white-tailed deer at Wyoming Game and Fish Department hunter check stations across the state of Wyoming. Samples were screened for BTV specific antibodies using ELISA assays and the positive samples then were

tested for specific serotypes using serum neutralization assays. For all wildlife species 8.4% (152/1805) tested antibody positive to BTV using the ELISA assays. Of the screened antibody positives, 27% (41/152) were BTV-11 positive, 88.2% (134/152) were BTV-17 positive. Some of these were positive for both serotypes indicating prior infection with both forms. Models were generated using backwards logistic regression to identify significant ($p<0.1$) predictor variables for both viruses. The risk maps for the model showed the highest risk of BTV seropositivity in the northeastern corner and along the eastern edge of the state and in the Bighorn Basin of Wyoming. The final risk model for BTV included the predictor variables of species and elevation, and landscape aspect and average summer precipitation were also significant.

KEYWORDS

Bluetongue virus (BTV), enzyme-linked immunosorbent assay (ELISA), epizootic hemorrhagic disease virus (EHDV), hemorrhagic disease (HD), mule deer (*Odocolileus hemionus*), pronghorn (*Antilocapra americana*), risk model (logistic regression), serum neutralization, white-tailed deer (*Odocoileus virgineanus*), Wyoming

INTRODUCTION

The viruses that cause bluetongue disease (BT) and epizootic hemorrhagic disease (EHD) are collectively known as hemorrhagic disease (HD) viruses (Williams and Barker, 2001). Both are closely related and belong to the family *Reoviridae*, genus *Orbivirus* and have a dsRNA genome with 10 segments. Bluetongue and EHD are vector transmitted, and cause noncontagious disease in wild and domestic ruminants including, domestic sheep (*Ovis aries*), domestic cattle (*Bos taurus*), white-tailed deer (*Odocoileus virgineanus*), mule deer (*Odocolileus hemionus*), pronghorn (*Antilocapra americana*), elk (*Cervus canadenisis*), and bighorn sheep (*Ovis*

canadensis). In Wyoming these viruses have been isolated from all these species (Afshar et al., 1995, Miller et al., 2010).

There are 24 recognized serotypes of bluetongue virus (BTV) worldwide with serotypes 1, 2, 10, 11, 13 and 17 in the United States (U.S.) (Mellor et al., 2008, Schwartz-Cornil et al., 2008, MacLachlan et al., 2009). Bluetongue serotype 17 is the common serotype historically found in Wyoming (Miller et al., 2010). Although there is some controversy over the number of epizootic hemorrhagic disease virus (EHDV) serotypes, it is generally accepted that there are 8 serotypes with EHDV-1, 2 and 6 present in the U.S. (Gaydos et al., 2002, Allison et al., 2012). Epizootic hemorrhagic disease virus serotype 2 is most often isolated from wildlife mortalities in Wyoming (M. Miller, Virologist, Wyoming State Veterinary Laboratory, Laramie, Wyoming, unpublished data) and in the U.S. (Nettles and Stallknecht, 1992). While both are causes of HD, there are differences in host susceptibility and geographic distribution between the two viruses.

Wild ruminants infected with HD viruses are commonly found dead with no overt signs of illness. In the peracute and acute forms of HD, animals are in good nutritional health but if observed ante mortem, may have a combination of fever, depression, inability to rise, respiratory distress, and edema of the head, neck, and tongue (Kreeger et al., 2011). Post mortem gross lesions of peracute disease include swelling and reddening of the conjunctiva, swelling of the tongue, and pulmonary edema (Kreeger et al., 2011). Lesions of acute disease include edema of the head, neck, and tongue, petechial and surface hemorrhage on many organs (heart, stomach, intestines), pulmonary congestion and edema, and oral erosion or ulceration. Testicular hemorrhage is also common (Williams and Barker, 2001). Chronic infection in wildlife is rare, but can occur and manifests as emaciated animals with chronic lameness due to hoof lesions

(Kreeger et al., 2011). In sheep BTV can cause severe clinical disease and death but is generally subclinical in cattle (Lefevre et al., 2010).

Bluetongue virus is transmitted by species of *Culicoides* (Diptera: Ceratopogonidae) biting midges (Nettles and Stallknecht, 1992, Williams and Barker, 2001). There are more than 1,400 species of midges that inhabit every major land mass except Antarctica and New Zealand and they are found at elevation from sea level to 4,000 meters in climates from tundra to tropics (Mellor et al., 2000). These 1 to 3mm long insects are among the world's smallest hematophagous flies. Worldwide these insects are responsible for the spread of over 50 viral diseases, most notably African horse sickness virus (AHSV), BTV, EHDV, equine encephalosis virus (EEV), Akabane virus (AKAV), bovine ephemeral fever virus (BEFV), and Palyam virus (Mellor et al., 2000). *Culicoides variipennis* is thought to be the primary vector for BTV and EHDV in North America (Mellor et al., 2000). With the recent division of this species into three subspecies (*C. v. sonorensis, C. v. occidentalis* and *C. v. variipennis*), *C. v. sonorensis* is now thought to be the primary vector of BTV (Mellor et al., 2000). With climate change and the ability to disperse long distances on the wind, it seems likely that *Culicoides*-borne diseases will spread into new regions potentially causing disease outbreaks in naïve populations. This has been demonstrated with the recent spread of new emerging *Orbiviruses* into Europe, including BTV-8 as far north as the Scandinavian countries (Burgin et al., 2013).

The most recent outbreak of BTV in Wyoming occurred in 2007 affecting both wildlife and domestic sheep (Miller et al., 2010). In early September 2007, BTV was reported in pronghorn, mule deer, and white-tailed deer across the state and caused morbidity and mortality in sheep flocks across the Bighorn Basin of Wyoming (Miller et al., 2010).

Serological tests for BTV include agar gel immunodiffusion (AGID), serum neutralization, and enzyme-linked immunosorbent assays (ELISAs). There is cross reaction between BTV and EHDV antibodies on AGID tests because of the similarities in viral proteins 7 and 3 for these viruses. Enzyme-linked immunosorbent assays are more specific with little to no cross reaction between groups (Lefevre et al., 2010). Agar gel immune diffusion and ELISAs are used for group specific antibody detection, and to determine the specific serotype, serum neutralization assays are needed (Williams and Barker, 2001). There are commercially available ELISA tests for BTV antibody detection. Virus isolation or polymerase chain reaction (PCR) can be used to detect the presence of virus or viral nucleic acid indicating a current HD infection (Williams and Barker, 2001). Serological tests detect antibody and are thus an indication of prior exposure (Lefevre et al., 2010). Blood filter strips have been successfully used for blood collection in many wildlife species and for detecting antibodies to viruses and bacteria including hepatitis E virus in wild Sika deer (*Cervus Nippon*) (Yu et al., 2007), *Brucella* in Caribou (*Rangifer tarandus*) (Curry et al., 2011), Newcastle disease in poultry (Beard and Brugh, 1977), and EHDV and BTV in white-tailed and mule deer (Stallknecht and Davidson, 1992, Dubay et al., 2006). This sampling method is a practical method for obtaining blood samples in field settings.

Of these two HD viruses, BTV is better researched, but less is known about EHDV. Much of our knowledge is based on in-vitro laboratory research, but the factors that contribute to natural outbreaks in the western U.S. are poorly understood (Williams and Barker, 2001). Hemorrhagic disease outbreaks occur in the late summer and early fall, typically below 2134 meters elevation, and in four to seven year cycles with minimal clinical disease in intermediate years (Kreeger et al., 2011). Animals are often found dead by water sources (Kreeger et al.,

2011). The vector breeding habitat is in the mud and standing water along the edges of water sources (Mellor et al., 2000). The geographic distribution of BTV across the state of Wyoming has not been clearly defined but documented outbreaks have occurred in the northeastern corner of the state. Most of the information about these diseases in Wyoming comes from morality events, so it is not clear if and where the virus circulates in years of little/no incidence, and if there are areas that have high exposure without severe disease. The project objectives were to 1) test hunter-harvested wildlife (pronghorn, mule deer and white-tailed deer) across the state for antibodies to BTV, 2) further characterize positive samples from objective 1 to determine if antibodies are serotype specific for BTV-11, BTV-17; and 3) develop geospatial models to predict risk of BTV infection in wildlife, using climatic and geographic selection parameters of elevation, slope, aspect, average summer precipitation, average maximum temperature, average minimum temperature, proximity to roads, proximity to water, and land cover type.

MATERIALS AND METHODS

Study Area and Sample Acquisition:

The study area was the state of Wyoming. Blood samples were collected from hunter-harvested pronghorn, mule deer and white-tailed deer at Wyoming Game and Fish Department (WGFD) hunter check stations during the fall seasons of 2011 and 2012 using Advantec® Nobuto Blood Filter strips (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The WGFD divides the state into herd units and hunt areas by species for hunting purposes. Herd units are grouped into one of eight regions with regional offices. Wildlife populations are managed on the herd unit level with population objectives set and adjusted in part by hunter harvest. Hunting licenses are issued by hunt area. Wyoming Game and Fish estimates populations of pronghorn, mule deer and white-tailed deer to be 407,600, 374,400, and 62,900 animals, respectively. The sample size

calculation was based on Peduzzi et al (1996) with ten positive samples required for each predictor variable to be evaluated in the model. Ten predictor variables were used so 100 positive samples were needed. As a low estimate, state-wide prevalence of five percent was assumed and 2,000 samples was the target sample size needed. Since the return rate on sample kits was expected to be low, 3,000 kits were distributed across the state each year giving each of the eight regions a set number of sample kits (Table 1) based on a weighted proportion of the total target population (hunter harvested animals) from the prior year WGFD Job Completion Reports harvest data. For the 2012 collection, the overall target sample numbers were rounded and adjusted to increase the number of deer sampled (Table 1). Sample kits included a data sheet (Figure 1) and a single Nobuto strip in a 5 mL culture tube (Genesee Scientific Corporation©, San Diego, California). Information requested on the data sheets included species, sex, approximate age, hunt area number, harvest date and location in UTM, latitude and longitude, GPS coordinates, or specific location description (ex. state section at the end of Ormsby road) . Sample kits were distributed to all eight regions with instruction sheets on sample collection (Figure 2) and a general letter explaining the research project. Blood samples were collected by immersing the narrow end of the Nobuto strip in pooled blood in harvested animals until the strip was saturated (approximately 10 seconds). The strip was then allowed to air dry (approximately 1 minute) and returned to the culture tube (with the lid left off) and placed in the plastic bag with the completed data sheet.

Sample Processing:

Samples were refrigerated at 4°C for no more than 2 days then completely dried at room temperature for 24 hours, the handle removed and remaining piece cut into 6 pieces (approximately 5 mm each) and stored at -80°C until the eluting steps. The Nobuto elution

protocol was developed from a combination of three protocols: Nobuto Manufacture instructions, the Wyoming Game and Fish Disease Research Laboratory protocol, and Stallnecht and Davidson (1996). Briefly, Nobuto strips cut into six pieces were eluted 1:10 by adding 400 uL Phosphate Buffered Saline (PBS) and stored at 4°C for 24 hours. A metal rod was used to pack the strip into the bottom of the tube before centrifugation at 3000g for 15 minutes. The eluate was then removed and placed in a new tube and stored at -80°C until testing.

Location data:

Harvest locations were reported in multiple forms (UTMs, lat/log, etc). Only location data that was Universal Transverse Mercator (UTM), latitude and longitude (lat/log) coordinate, Public Land Survey System (PLSS) including the township, range and section, or detailed description on the landscape with quality landmarks was used. For PLSS section level data, random points were generated using mapping software (Wyoming 2011 All Topo Maps V6, iGage Mapping Corporation, Salt Lake City, Utah) to determine the corner coordinates (an easting and northing for each) for each of the four corners for the given section (640 acres) in a township and range. Since sections are not always perfectly square, a median of each easting and northing for each of the four sides was calculated in Microsoft Excel resulting in two easting and northing values for each section. Then a random function picked a single easting and northing coordinate. The randomly generated coordinates were imported back into the mapping software to determine position on the landscape. Points that landed in bodies of water, outside the designated section, or in the middle of improved roads and highways were discarded and the next randomly generated coordinates were selected. This process was repeated until a valid random point in the given section was determined.

Antibody Testing: ELISA and Serum Neutralization

Samples were tested for BTV antibody using a competitive ELISA (cELISA) (VMRD, Inc., Pullman, Washington, USA) following manufacturer's instructions. Test eluates producing an optical density (O.D.) greater than or equal to 50 percent of the mean of negative controls were deemed negative and test eluates that produced an O.D. of less than 50 percent of the mean of negative controls were considered positive. Known BTV antibody positive and negative samples were tested on every plate.

Method validation was performed for the BTV cELISA since the manufacturer's instructions called for the use of sera while our samples were serum eluted from Nobuto strips. Domestic sheep sera (n=10, 6 seropositive and 4 seronegative) were tested in duplicate with paired serum eluted from Nobuto strip.

Serum neutralization assays were used to determine serotype for samples that tested BTV antibody positive by cELISA. Briefly, 20 uL of sample eluate was diluted 1:10 in 4% Minimal Essential Medium (MEM) of cell culture media (Gibco®, Life Technologies, Carlsbad, California) and incubated at 57°C for 30 min before being combined with an equal volume of medium containing 100 tissue culture infective dose 50 ($TCID_{50}$) of the virus. The eluate and virus were incubated at 37°C for 30 min, and then added to duplicate wells of a 96-well plate with confluent African green monkey kidney cells (Vero 76, ATTC®, Manassas, Virginia). Eluate and virus were incubated at 37°C on the corresponding cell lines for three hours, before removal and replacement of complete medium. The plates were then incubated at 37°C for five to seven days until cytopathic effect (CPE) was observed in virus infected and negative control wells. Wells were then stained with crystal violet stain and test results were determined as positive by >50% neutralization of CPE.

Model development and statistical analysis:

Species, elevation, slope, aspect, average summer precipitation, average maximum and average minimum temperature, proximity to roads and proximity to water, and land cover type were selected for evaluation as predictor variables. Aspect values range from 0 to 359.9 and are measured clockwise from north (0 degrees). Aspect values were classified based on ranges of the values of the compass direction were flat (-1 degrees), north (315-44 degrees), east (45-134 degrees), south (135-224 degrees), and west (225-314 degrees). Land cover was classified as grassland (including prairie and non-irrigated crop land), shrub land, forest, wetland (including riparian and irrigated crops), and settlement. Statistical Analysis Software (SAS©; SAS Institute Inc., Cary, North Carolina) was used for all statistical analysis and ArcGIS 9.2 (ERSI, Redlands, California) was used for mapping. A correlation analysis (proc corr) was conducted to determine a predictor variable correlation coefficient. Predictor variables with a Pearson's correlation coefficient of greater than 0.8 were considered highly correlated and considered for removal from the model. Stepwise backwards elimination (proc logistic / backward) was conducted to systematically eliminate variables based on the specified significant p-value of <0.1. Briefly, the predictor variable with the highest p-value was eliminated and the model was re-run without this predictor variable. The predictor variable with the second highest p-value was then removed and the model was re-run without this predictor variable and the process was continued until only significant ($p < 0.1$) predictor variables remained in the model. Then a re-entry criterion of $p< 0.25$ was chosen so all eliminated predictor variables were then considered for re-entry into the model based on this criterion. If a predictor variable met this criteria, it was re-entered and the model was re-run and the predictor variable was evaluated for elimination based on the original elimination criterion ($p <0.1$). This process continued until there were no predictor variables that

met the criterion for addition to or elimination from the model and subsequently the final model was produced. A logistic regression (proc logistic) was conducted to compare model agreement with the backwards elimination significant predictor variables. A final model was selected for each BTV and EHDV. Estimate values for significant predictor variables were used in model equations in ArcMap raster calculator to create a probability surface that was used to generate risk maps for BTV and EDHV.

RESULTS

ELISA Validation:

For the BTV cELISA domestic sheep sera (n=10, 6 positive and 4 negative) was tested in duplicate with paired sera eluted from Nobuto strip and all (10/10) eluates gave identical results to the paired sera.

Antibody Testing: ELISA and Serum Neutralization Results

Across all three species 8.4% (152/1805) had detectable BTV antibody, with pronghorn 8.7% (69/723), mule deer 8.5% (70/757), and white-tailed deer 7.0% (13/173) testing positive. Of the 152 samples that tested BTV positive on the BTV ELISA, 27% (41/152) had BTV-11 antibody, 88% (134/152) were found to be BTV-17 specific, and 11% (16/152) did not neutralize (Table 2). The location of sample collection and positive reactions are illustrated on a map of Wyoming in Figure 3.

Modeling and Statistics:

Pearson correlation coefficients did not exceed 0.8, so all variable were analyzed in the backwards elimination logistic regression. For the BTV model the predictor variables of species, year, age, elevation, aspect, and average summer precipitation were significant ($p<0.10$) (Table 3).

In the BTV final model, elevation was protective. With every meter increase in elevation there was a decrease in BTV antibody risk. Additionally the predictor variables of aspects north and west were also protective for being antibody positive. On the other hand, flat aspects were the most risky followed by east facing slopes. Furthermore pronghorn were the highest risk species, while being a white-tailed deer was protective for being antibody positive. Predictor variables found to be significant are presented in Table 3. Risk maps were generated based on the model that illustrate the distribution for the three wildlife species (Figures 4-6) with the high risk areas including the northeastern quadrant of the state and extending down the eastern edge of Wyoming. On the risk maps, low risk areas (areas of dark green on maps) follow the lines of mountain ranges in the state.

DISCUSSION

Positive samples from the BTV specific ELISA tests were further tested by SN assays for known or suspected BTV serotypes in Wyoming. The majority of these were found to be BTV-17 (88%) as expected, but we also found that BTV-11 is present in the state with an overall prevalence of 27%, and some samples were positive for both serotypes (Table 2). Some of the samples did not neutralize either BTV-17 or 11. This is most likely due to the relatively unclean samples possessing toxic components to the cell cultures causing cytopathic effect not induced by virus, however we cannot rule out the possibility that a serotype other than BTV-17 or 11 was the source of infection. In the summer of 2012, BTV-10 and 13 were both reported in north-eastern Colorado so these serotypes may also be present in Wyoming. Future work could include testing samples for other serotypes present in neighboring states.

We demonstrated an uneven distribution of BTV antibody positive wild ungulates across Wyoming with the highest number of positive wildlife in a triangular area east of a diagonal line from the middle of the northern border running diagonally to the southeastern corner. This distribution is illustrated by the generated risk maps by species (Figures 4-6) with the high risk areas including the northeastern quadrant of the state and extending down the eastern edge of Wyoming. This distribution of risk for antibody positive status corresponds with regions with previously reported BTV outbreaks, and anecdotal reports from landowners, wildlife biologists, hunters and game wardens of significant morbidity and mortality.

In the BTV model, elevation and average summer precipitation were significant predictor variables that were expected. Higher elevation areas of Wyoming are not ideal midge habitat and fewer (if any) vectors in these ecosystems clearly is associated with a decreased risk of BTV transmission. In years with more precipitation there is more standing water and thus an increase in the amount of midge breeding habitat (Mellor et al., 2000) – this again occurs most often at lower elevations in Wyoming. The predictor variable of aspect was an unexpected finding, with flat surfaces associated with the highest risk of being BTV seropositive. One explanation is that flat areas are more likely to be low elevation areas with standing water thus being more ideal *Culicoides* habitat. The next most risky aspects were east and south respectively. Interestingly two aspects (north and west) were protective against BTV antibody.

In this model antibody positive is used as a proxy for infection so proportions of the population that do not survive infection are not included in these data. Wildlife in the high risk regions are more likely to develop detectable antibody to BTV, but they must survive infection and be harvested by hunters to be included in our sampling. The risk model was based on seropositivity so it would appear that white-tailed deer are at lower risk of HD viruses compared

to pronghorn and mule deer. However, it can only be stated that in Wyoming white-tailed deer are less likely to have detectable antibody to BTV. Since HD outbreaks around the country in white-tailed deer have resulted in high mortalities (Williams and Barker, 2001) it seems probable that they are less likely to survive viral infection, thus there are fewer antibody positive animals in the population resulting in a seemingly lower risk for infection.

This research provides baseline information for BTV distribution and geographic and climate risk factors for infection in Wyoming that can be used to guide areas for future research. Our findings confirmed the general understandings of the importance of elevation, precipitation and temperature to HD Wyoming, but also suggest the novel possibility that aspect of the landscape may be involved in the disease ecology. It is interesting to hypothesize that north and west facing slopes provide protective features such as cooler temperatures and varied exposure to prevailing winds, but further research is needed to verify this finding and to determine possible mechanisms.

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Table 1: Target sample collection numbers by region in 2011/2012 and the number of samples tested for BTV antibody. Year of sample collection by year is presented as 2011/2012

	Pronghorn	Mule Deer	White-tailed Deer	Total
Casper	232/100	212/200	409/200	853/500
Cody	29/25	169/75	134/100	332/200
Green River	177/50	98/100	0/0	275/150
Jackson	0/0	68/50	0/0	68/50
Lander	94/50	83/100	0/150	177/300
Laramie	257/50	113/125	42/125	412/300
Pinedale	0/0	57/50	0/0	57/50
Sheridan	210/100	199/200	414/200	823/500
Total Collected (2011 + 2012)	**1060**	**909**	**360**	**2329**
Total Tested (2011 + 2012)	**792**	**827**	**186**	**1805**

Table 2: Bluetongue virus antibody detection and serotype distribution.

Species	Percentage Bluetongue Virus Antibody Positive
Pronghorn	8.7 (69/792)
Mule Deer	8.5 (70/827)
White-tailed Deer	7.0 (13/186)
Total	**8.4 (152/1805)**
Total BTV-17	88.2 (134/152)
Total BTV-11	26.9 (41/152)
Total neither BTV 17 nor 11	10.5 (16/152)

Table 3: Predictor variable estimates for the BTV model and standard errors. Asterisks denote classified variables.

	BTV Model	
Predictor variable	Log (Odds)	SE
Species pronghorn*	0.4550	0.1455
Species mule deer*	0.2682	0.1428
Species white-tailed deer*	-0.7232	
Elevation	-0.00246	0.000313
Aspect flat	0.6470	0.1837
Aspect north*	-0.3797	0.1858
Aspect east*	0.1683	0.1563
Aspect south*	0.0363	0.1756
Aspect west*	-0.4719	
Average Summer Precipitation	0.7644	0.2880
Average Maximum Temperature	-	-
Proximity to Water	-	-

University of Wyoming- Department of Veterinary Sciences

Dear Hunter,

Bluetongue (BT) is an infectious, vector-borne, noncontagious disease of wild and domestic ruminants including, white-tailed deer, mule deer, pronghorn, elk, and bighorn sheep. It is transmitted by *Culicoides* species biting midges. The information collected from this brief questionnaire in conjunction with the blood sample will help identify environmental and spatial factors that contribute to BT in Wyoming wildlife. Please fill out the information below as thoroughly as possible. Thank you for taking the time to complete this information.

SPECIES: Pronghorn ☐ Mule Deer ☐ White-tailed Deer ☐ **SEX:** Male ☐ Female ☐

APPROXIMATE AGE: fawn ☐ yearling ☐ adult ☐

HUNT AREA NUMBER: ________________ **DATE HARVESTED:**__________________________

LOCATION OF HARVEST: (*be as specific as possible;* lat/long, UTM, township/range/sections, ranch name)

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Thank you again for your help.

Figure 1: Hunter-harvested data collection sheet included in all test kits and filled out by WGFD personnel at hunter check stations.

Blood sampling with filter paper strips

1. Dip **narrow end** of the strip in pooling blood
2. Allow to air dry
3. Place in tube
4. Place tube in the bag along with a completed questionnaire

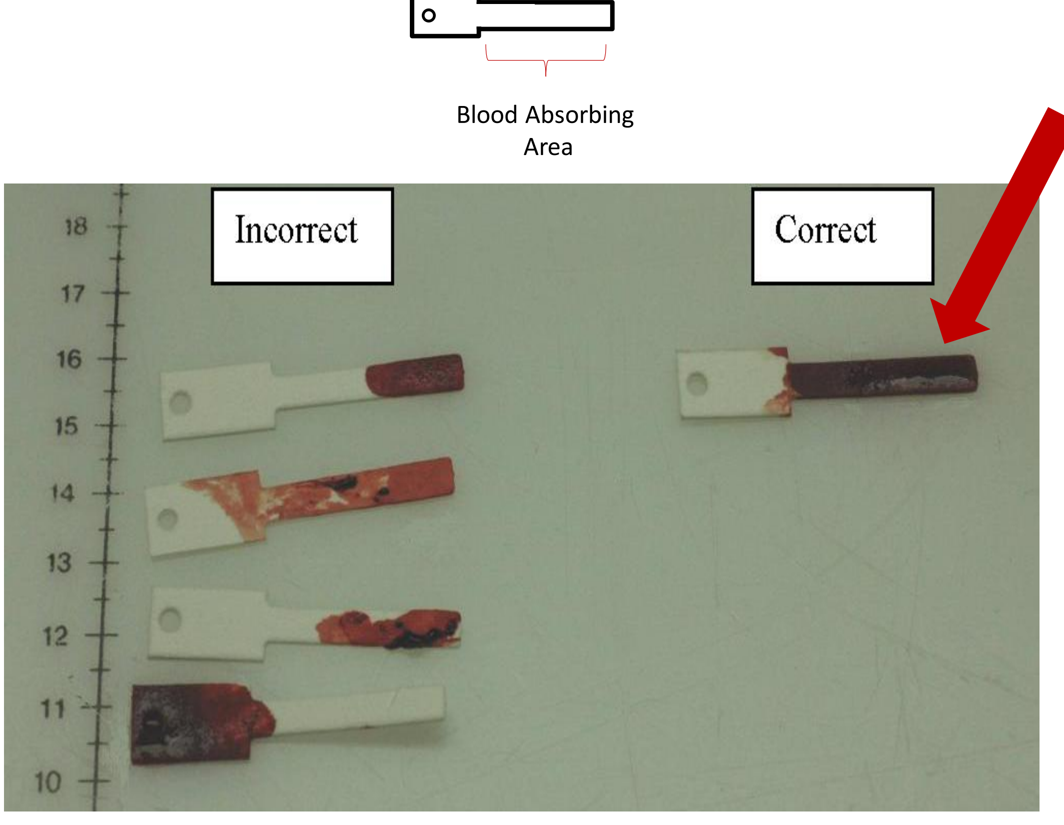

Figure 2: Bluetongue test kit Nobuto blood filter strip sampling instructions.

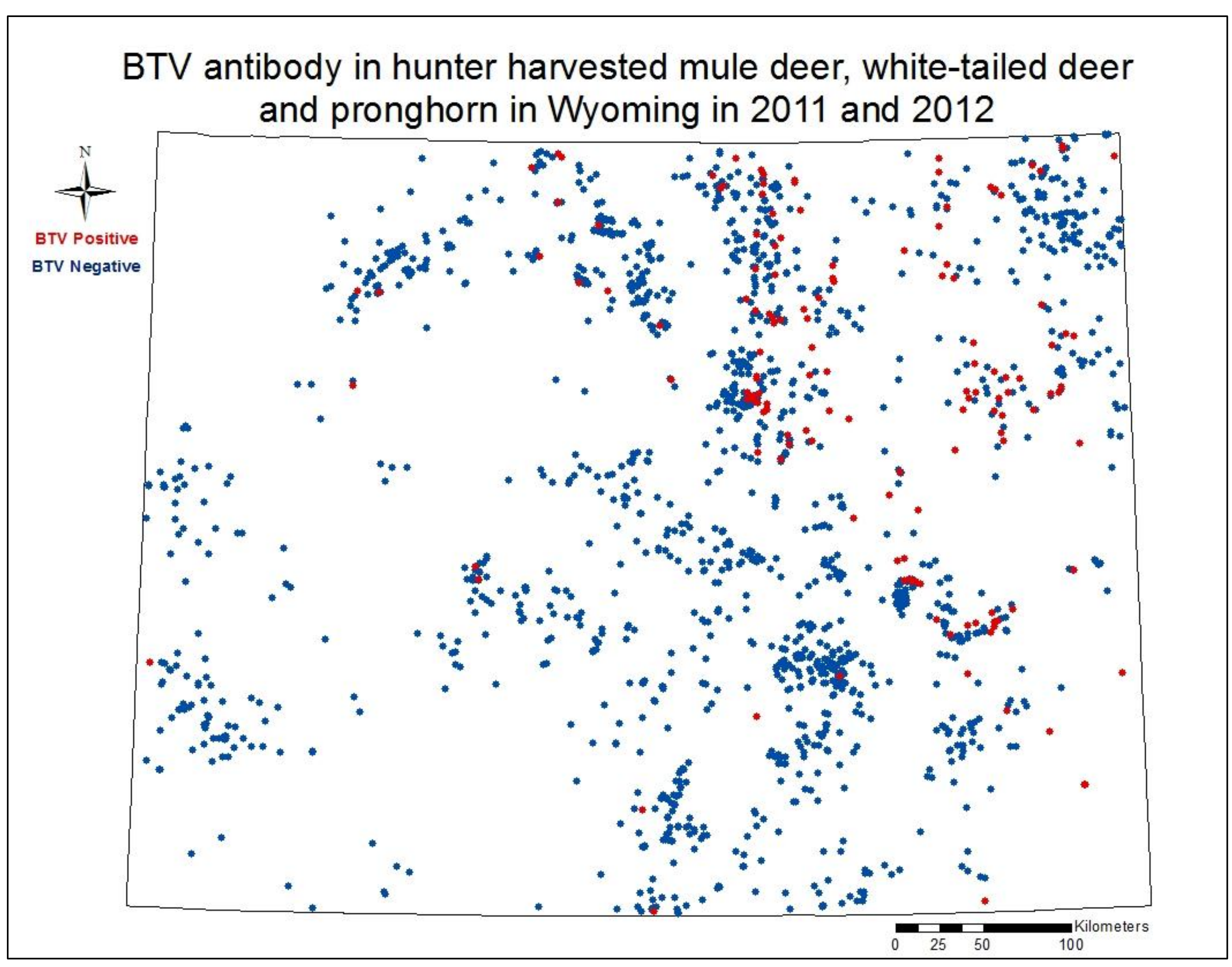

Figure 3: Harvest locations of pronghorn, mule deer and white-tailed deer with corresponding BTV test result.

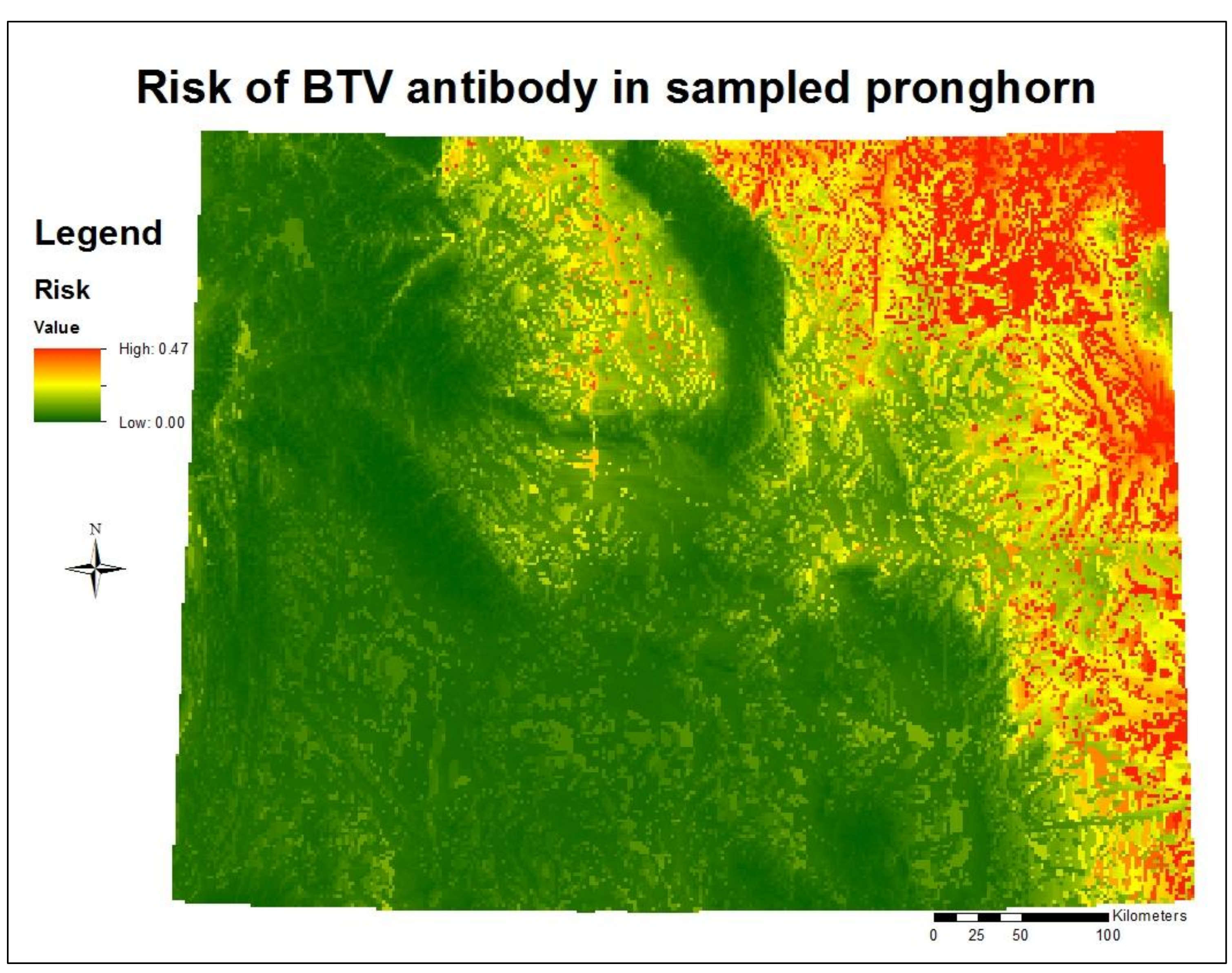

Figure 4: Risk map of bluetongue virus antibody in pronghorn. Scale displayed from 0 to 0.47 not 0 to 1.

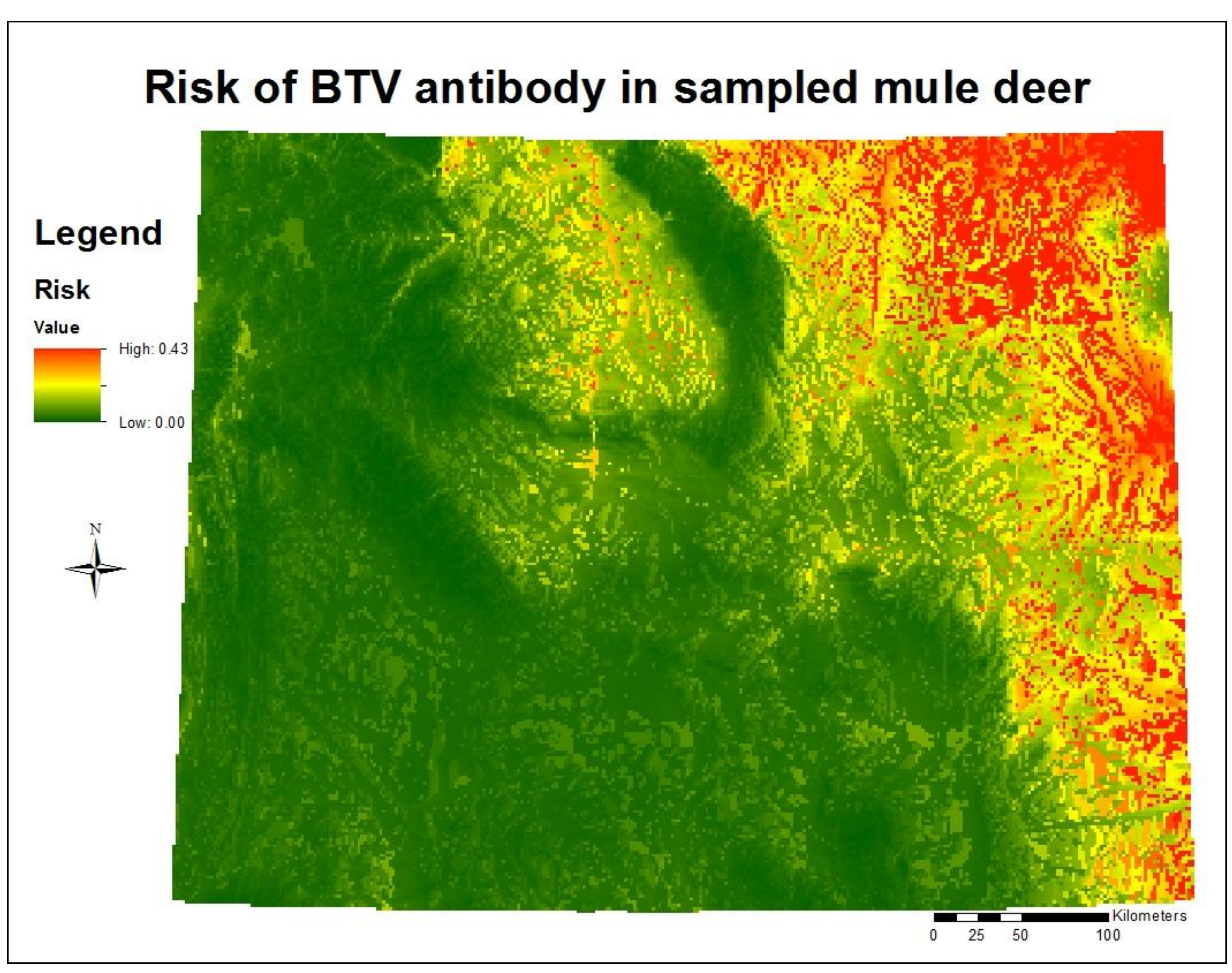

Figure 5: Risk map of bluetongue virus antibody in mule deer. Scale displayed from 0 to 0.43 not 0 to 1.

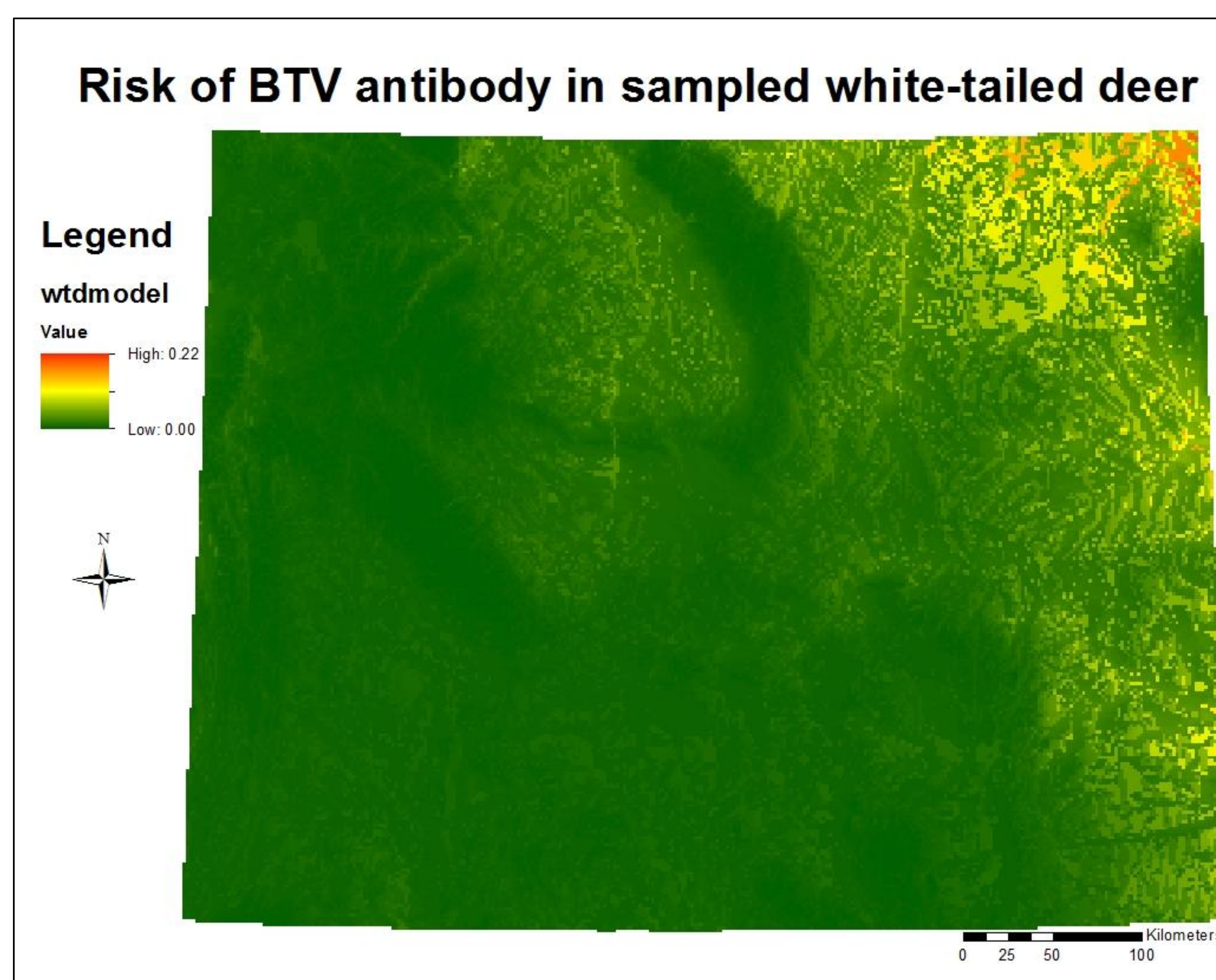

Figure 6: Risk map of bluetongue virus antibody in pronghorn. Scale displayed from 0 to 0.22 not 0 to 1.